Organ Preservation into the 2020's:

the Era of Dynamic Intervention

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Abstract:

Organ preservation has been of major importance ever since transplantation developed into a global clinical activity. The relatively simple procedures were developed on a basic comprehension of low temperature biology as related to organs outside the body. In the past decade, there has been a significant increase in knowledge of the sequelae of effects in preserved organs, and how dynamic intervention by perfusion can be used to mitigate injury and improve the quality of the donated organs. The present review focuses (1) on new information about the cell and molecular events impacting on ischaemia / reperfusion injury during organ preservation; (2) strategies which use varied compositions and additives in organ preservation solutions to deal with these; (3) clear definitions of the developing protocols for dynamic organ perfusion preservation; (4) information on how the choice of perfusion solutions can impact on desired attributes of dynamic organ perfusion; (5) summary and future horizons.

1. Introduction

Organ transplantation has developed over the past 60 years from what could be considered as a surgical research technique available to only a handful of patients into a globally accepted treatment for end organ failure [1–3]. Many advances have been made in clinical understanding of the essential elements for success, including patient management, specialised surgical intervention, immunosuppression, and, of note in this review, in organ preservation which has been considered as the supply line for clinical transplantation [4, 5]. In this review we will consider (1) the history and current applications of organ preservation; (2) new achievements in our understanding of the ischaemic injury which can result during donor organ retrieval, and how these might be mitigated using new additives to organ preservation solutions; (3) the scientific basis for machine perfusion of donor organs which is becoming a new focus in the organ preservation pathway.

2. Historical Perspectives of Organ Preservation for Transplantation

A central focus for organ preservation has been to match the need to transport donor organs from the centre where surgical retrieval has been performed to the implanting centre, which may be many kilometres distant, and the potentially harmful functional consequences of hypoxia once the organ has been deprived of normal blood supply. Cooling is an intuitive way to reduce metabolic demand, but applying it to organs *ex vivo* and in a ways which could be readily reversed on transplantation, across a range of abdominal and cardiothoracic organs, required considerable research and development. Early in the 20th century, attempts to produce artificial circulatory support to perfuse organs *in vitro* were developed in systems such as the kidney and liver by simple syringe methods, and provided detailed information on physiology of organ function [6]. In parallel, efforts were made to formulate synthetic perfusate solutions of electrolytes, solutes, vitamins etc, capable of replacing blood in some respects [7, 8]. At the same time, information on the impact of hypothermia on organ function was also starting to be recorded [9].

Early investigations on the use of hypothermia in organs subsequently transplanted was undertaken by Calne et al. in the kidney. They investigated the relative merits of cooling kidneys by simple surface cooling or by perfusion of the renal artery with cooled heparinised blood [10] and concluded that vascular flush-perfusion was the preferred method of cooling. However, the use of diluted blood in this way still led to problems with early vascular stasis in the grafted kidney, promoting the search for better organ preservation solutions (OPS), preferably synthetic solutions which could be reliably manufactured and sterilised to be available at short notice as required [11]. The OPS described by Collins, and his colleagues Bravo-Shugarman, and Terasaki [12] was designed to mimic, in a simple fashion, the

intracellular electrolyte balance, which was in turn based on an understanding of the ongoing changes in cells during hypothermic and hypoxic exposure. This solution became a mainstay of clinical organ preservation for almost two decades. Successful, renal preservation was achieved for up to one day, long enough to allow transplant tissue matching and sharing of organs over a wide geographic area. Alongside these approaches, proponents of continuous hypothermic machine perfusion continued to develop methods for use of bloodless perfusates in oxygenated low temperature perfusion across a range of organs [13–15]. However, the logistics and reliability of the available equipment meant that gradually, flush cooling and ice-storage with synthetic OPS became the most widely used preservation method and allowed techniques for multiple organ retrievals from a single donor to be developed using the so-called 'flexible techniques' [16] in which all organs designated for transplantation are cooled in situ, rapidly removed in a bloodless field, and further dissected on a back table. Following on from Collins' work, other formulations of commercially-produced OPS came forward based on progressive research into organ cold hypoxia. For example, Ross, Marshall et al. [17] developed an intracellular mimick based on citrate as the major anion. Later, Belzer, Southard and colleagues [18] proposed another formulation with improved buffering and anion composition, which became known as University of Winsconstin (UW) solution and remains in wide clinical application today. These will be discussed in greater detail below.

Since that time, it will be apparent that there is a fundamental choice to be made in organ preservation; whether to sustain *ex vivo* circulation by hypothermic machine perfusion (HMP) and to supply oxygen to sustain low-level oxidative organ metabolism, or whether to use OPS to protect against the progressive hypoxia in the isolated organ during static cold storage (SCS) whilst using inexpensive and readily transportable ice storage of the cold-flushed, sterile packed organ. For many years, the choice of SCS has dominated clinical organ preservation, but currently there is growing interest once more in dynamic intervention by organ perfusion, driven by many factors including changing donor demographics and the need to utilise all available organs for transplantation to meet rising clinical need. Recent publications continue to debate these issues [19–21].

3. Ischaemia Reperfusion (I/R) Injury as a Major Focus in Organ Preservation

3.1. Regulatory mechanisms of cellular injury

Historically, the development of effective OPS has always been accompanied by a deep focus on the mechanisms and strategies to attenuate I/R–induced cellular injury.

Preservations methods and choice of solutions have an impact on organs across a diverse range of molecular response, thus providing crucial information about the timelines of processes to develop injuries and their physicochemical variations in relation to the OPS used (see section 4 for more detail). In this case, special importance is given to the cellular and intracellular machineries under the pathological conditions of hypoxia. On the one hand, the cellular compartments are compromised by the duration of the ischaemic period, donor age, trauma, existing infection or other factors. On other hand, cellular heterogeneity, reflecting the organ complexity influences profound imbalances in metabolism during I/R. The studies performed over the last 3 decades using isolated cell systems have significantly broadened our understanding of I/R, yet many pathophysiological aspects of the intercellular cooperation in tissue are still far from a unified understanding.

Experiments with I/R clearly demonstrated that severity of the injury after reperfusion directly correlated with the interval of ischemia. Hence, restoration of blood supply to the organ in the shortest possible time is essential. The general paradigm of the cellular reactions upon I/R may reflect a picture of the sterile inflammation phenomenon [22]. This inflammation is predominated by the sentinel pattern recognition receptor (PRR) systems [23] and involves complex reactions such as reactive oxygen species (ROS), leukocyte and platelet sequestration to the endothelium [24], transmigration of neutrophils, and the release of endogenous inflammatory mediators.

3.2. Microvascular dysfunction and immune cells response in I/R-induced inflammation

Microvascular dysfunction is an early factor in the pathogenesis of I/R injury and forms the basis for elaborating the underlying mechanisms of this pathological process. This type of injury is most frequently associated with SCS. Studies of rat liver transplantation demonstrated the crucial role of the onset of the blood cessation in development of I/R injury, bringing into question early microvascular impairment as a prognostic parameter for the assessment of early graft function in clinical practice [25]. It has been demonstrated that even a short duration of 20 minutes warm ischemia in the liver results in decreased sinusoidal diameter which is associated with ’plugging’ of leukocytes upon reperfusion, as well as sinusoidal obstruction caused by endothelial cell swelling [26]. In contrast, cold ischemia protects from hepatic microcirculatory perfusion failure after 90 minutes of lobar ischemia [27]. However, prolongation of hypothermia to 24 hours significantly reduced sinusoidal perfusion rates [28].

The mechanical and functional basis of microvascular reflow impairment after I/R was attributed to a series of interrelated events which share common characteristics in different

organs. These include: interstitial edema formation due to increased capillary permeability [29], when interstitial tissue pressure may physically compress the capillaries, and [30] microvascular spasm, caused by vasoactive substances.

Some preservation solution compositions impart a vasoactive property and affect tissue microcirculation during I/R. For instance, flushing the organ with low-potassium medium prior to preservation may protect microvessels from the adverse effects of cold storage and improve reperfusion after transplantation, in situations where vasoconstriction might be caused by a high-potassium content in the OPS. Liver flushing with the OPS, histidine–tryptophan–ketoglutarate (HTK), which is known to be low in K^+ ions, prior to organ storage in UW improved hepatic microcirculation and reduced sinusoidal leukostasis [31]. Another approach shows that a rinse with the low-potassium solution after cold storage prior to organ implementation substantially diminished postoperative sinusoidal endothelial cell damage. It was concluded, that synergistic action of UW as OPS and the Carolina Rinse solution as a rinsing vehicle, was more beneficial for the vascular bed than each solution used separately [25, 32]. This finding provides some arguments in favor of removal of high-K^+-containing solution before transplantation.

The ‘no-reflow’ phenomenon in I/R is attributed to leukocytes plugging of the capillary lumens. Leukocytes are large and stiff cells, which adhere to vascular endothelium are more likely to become captured in capillaries, thereby obstructing luminal flow. Heart and kidney I/R models have demonstrated the presence of leukocytes in a very high proportion in occluded capillaries. Additionally, the impact of hemodynamic forces upon reperfusion has also been implicated in the adhesion of leukocytes and further impairment of endothelial cells [33]. Perfusing the organ with hypertonic saline-dextran solution decreased endothelial cell swelling, inhibited neutrophil adhesion [34] which can form part of therapeutic strategies to restore the normal capillary network [35].

Discovering the role of gas mediators such as hydrogen sulfide (H_2S), carbon monoxide (CO) and nitrous oxide (NO) is regarded as a milestone in organ preservation and transplantation and are promisingly considered to be effective in the protection of the endothelium during organ storage. In particular, across the wide spectrum of signaling functions, these molecules came out to be potent anti-platelet adhesion and anti-inflammatory substances [36], modulating the relaxation of stellate cells and improving the microcirculation of hepatic sinusoids [37]. It has recently been shown, that endogenous H_2S regulates many physiological processes, including vascular tone. Additionally, H_2S was shown to interact with NO and form another potent vasorelaxant - nitroxyl (HNO) [38].

After leukocyte plugging, the adhesion cascade is another major molecular process of leukocyte recruitment during an inflammatory response in I/R. Leukocyte adhesion with postcapillary venular endothelium initiates by forming loose contacts (leukocyte rolling). The sticking of leukocytes to the endothelium is mediated primarily by transmembrane adhesion receptors – integrins, selectins, vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1) – responsible for adhesion of cells and extracellular matrix and rolling. Recent studies have challenged the potential therapeutic significance of α4β1 integrin receptors (β1 integrin family), which binds with the connecting segment-1 (CS-1) – a V region of fibronectin. The latter is a key extracellular protein expressed by sinusoidal endothelial cells in the early beginning of liver ischaemia [39]. Blockage of α4β1-CS-1 region interaction with a specific peptide resulted in an increase in survival (from 40% to 100%) for 14 days after orthotopic liver transplantation as well as a reduction in TNF-α and IFN-γ expression, T-lymphocyte and leukocyte sequestration [40].

Interactions between integrin receptors [41] and other adhesion molecules like VCAM-1, ICAM-1, P- and E-selectins reveal a complicated and tissue-specific regulatory mechanism of leukocyte adhesion and extravasation. For instance, in comparison with lungs and heart, liver sinusoidal endothelium neutrophil tethering and 'crawling' does not exhibit strict dependency of P-selectin/integrin assistance [42, 43]. However, neutrophil extravasation from the hepatic microcirculation into the parenchyma is facilitated by β2 and β1 integrins [44], showing their direct contribution to ligands/counter-receptors participation in neutrophil transmigration. Interestingly, neutrophil extravasation through pericyte gaps into the parenchyma corresponds to regions with low contents of specific matrix proteins in the basement membrane. It could be hypothesised that the cold-induced decrease in endothelial junctional proteins such as F-actin, occludin and VE-cadherin [45] may be responsible not only for the development of organ oedema, but also in extravasation of neutrophils [46].The influence of OPS on the adhesion molecules expression could provide additional information in relation to recruitment of immune cells in inflammation during organ reperfusion. Comparative studies of HTK and UW solutions have shown much lower expression of P-selectin in UW group which demonstrates a significant benefit over HTK [47].

3.3. Kupffer cells and macrophages in I/R

Kupffer cells are resident macrophages of hepatic sinusoids which are well known to be a powerful source of ROS, proteases, platelet activating factor and cytokines during I/R. Activated Kupffer cells begin to produce pro-inflammatory cytokines, such as IL-1β and TNF-α, promoting the migration of neutrophils and CD4+ T lymphocytes with the subsequent development of inflammation in the parenchyma.

A growing body of data demonstrate that Kupffer cells are an important participant in the development of sterile inflammation mediated via toll-like receptors (TLRs), which are a significant part of signaling pathways in sentinel cells. In contrast to pathogen-activated inflammation, TLR are triggered by intracellular factors and fragments of extracellular matrix, named DAMPs (damage-associated molecular patterns) which are released from ischaemically injured cells. DAMPs are recognised by the innate immune cells (macrophages, leukocytes and dendritic cells) as well as vascular cells (fibroblasts and endothelial cells) to promote pro-inflammatory and profibrotic pathways [48].

DAMPs include high-mobility group box 1 (HMGB-1), DNA of mitochondria and nucleus, purine metabolites, hyaluronan and others. Despite the fact that precise regulatory mechanism of Kupffer cell activation is yet to be fully unveiled, the current experimental data amply demonstrate interrelation between Kupffer cell/TLR ligation and subsequent complement activation, ROS formation, neutrophil recruitment and platelet aggregation. The prominent role of TLR-4 triggering was confirmed in orthotopic liver transplantation models. Knockout mice deficient in liver TLR-4 had significantly lower liver expression of TNF-α, myeloperoxidase (MPO), aspartate aminotransferase (AST) and CD4+ T cell infiltration [49, 50]. In a model of renal inflammation, ischaemia-induced synthesis of TLR-2 and TLR-4 was substantially attenuated by inhibition of cytokine production with IFN-γ and TNF-α antibodies pretreatment. Similarly, inhibition of HMGB-1 – a nuclear and cytoplasmic ligand for TLR-2 and TLR-4, decreased liver injury after cold storage and warm reperfusion [51]. This feature demonstrates the possible reciprocal regulation of pro/anti-inflammatory factors and TLRs. Translation of inflammatory signals from TLR sustains feedback with target cells, resulting in suppression or activation of TLRs expression. Future research in TLRs function can provide a new basis for the development of ligand-receptor mediated drugs for limiting I/R injury.

Kupffer and other immune cells in the inflammatory response caused by either warm or cold ischemia display a high regulatory plasticity. In addition to ROS and cytokine production, there is evidence of the anti-inflammatory function of macrophages and Kupffer cells in heme-mediated injury. The scavenging of free hemoglobin from plasma is controlled by expression of the CD163 receptor on the surface of macrophages and monocytes. The endocytosis of hemoglobin via CD163-dependant internalisation also stimulates the production of heme oxygenase-1 (HO-1) in macrophages followed by degradation of toxic heme [52, 53]. The HO-1-induced breakdown of hemoglobin is accompanied by carbon monoxide release, known to possess strong anti-inflammatory and proangiogenic properties [54]. Another important aspect of Kupffer cells is induction of apoptosis and removal of PMNs during the tissue regenerative process [55]. Consequently, similar to other macrophages

[56], Kupffer cells play a dual role; in early reperfusion they intensify and at the later stages subside the inflammation to assist tissue healing.

3.4. The role of platelets in organ I/R injury

Platelet aggregation during haemostasis is regularly described as a privileging process of blood coagulation in the vascular defense against trauma. However, the decrease in platelet numbers due to their sequestration in organs after cold storage has apparently been considered as a potential risk in organ transplantation. A closer look at this problem shows a lack of evidence for platelet adhesion to vascular endothelium [57]. For example, after 24 h cold storage of liver in UW and subsequent normothermic reperfusion, there were no obvious clot formations or vessel occlusions observed. Therefore, it has been concluded that platelets-induced endothelial cell injury is probably not related to their procoagulant properties.

During past decades, information about the role of platelets in I/R injury has been revised and supplemented. There is compelling evidence to suggest that platelets participate in endothelial cell adhesion in cooperation with neutrophils and lymphocytes to initiate innate immune responses under different pathological conditions. Activated platelets were shown to stimulate neutrophils/macrophages ROS production, control endothelial permeability, and promote neutrophil infiltration. The hallmark of platelet-endothelial cell adhesion is the formation of a matrix which includes neutrophil extracellular traps (NETs), and P-selectin mediated leukocyte binding [58] that predominantly has negative consequences for the transplant functional state.

Activated platelets express a range of adhesion receptors, such as P-selectin (CD62P), TGFβ, PF4 (CXCL4), IL-1β and others, which predispose their interactions with immune cells, endothelial cells and adhesion molecules. The list of platelet receptors as well as a sequence of events, including mechanisms for their recruitment is constantly being updated [59]. The recent finding of HMGB-1 protein in platelets shows their importance in the regulation of NET formation and thrombosis [60] mediated via Toll-like receptors.

These varieties of signal transduction clearly encompass far more than the haemostatic or inflammatory functions of platelets, and also expand the horizon for endeavors in the treatment of organ I/R injury. For example, the translational studies of regeneration and repair post I/R suggest the participation of platelets in liver recovery after partial hepatectomy. The repairing effect of platelets could possibly be explained by contact or even uptake of cells by hepatocytes [61] and the release of insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and serotonin

from platelets, which together can promote hepatocyte proliferation [62]. Yet, further research is required to fully clarify platelet regenerative potential following organ I/R.

It may be expected that platelet aggregation and adhesion originates not only from pathological changes in themselves. Cold-induced alterations of the microvascular environment may readily contribute to platelet activation. The direct contact with collagen of exposed subendothelial extracellular matrix, ADP release from damaged cells or due to inhibition of T-lymphocyte ectonucleotidase, leukocyte cytokine production may be considered in the frontline of platelet adhesion and aggregation [63]. The balance between ROS and NO production serves as an alternative regulatory mechanism of platelet adhesion. It is extensively recognised that production of NO by endothelial cells prevents platelets adhesion. On the contrary, superoxide anion ($O2^-$) induces vascular adhesion of platelets and leukocytes. It is intriguing, that NO can scavenge superoxide anion much faster than superoxide dismutase does. Therefore, $O2^-$ overproduction after I/R may increasingly surpass NO bioavailability. Additionally, as was reported recently [64], modifications of endothelial NO synthase (eNOS), due to the loss of enzyme co-factor- tetrahydrobiopterin or/and eNOS uncoupling, makes production of ROS instead of NO possible.

Taken together, it is still controversial which of the factors predominate in platelet activation response. The understanding of the synergistic ways of intercellular regulation after cold storage will be essential for prevention of I/R injury [58].

3.5. The role of endothelial glycocalyx in I/R injury

Degradation of the endothelial glycocalyx (GCX) has been implicated in several disease processes including sepsis, trauma and I/R injury [65]. The GCX is a thin (60 to 570nm), fragile and ubiquitously expressed ‘hair-like’ layer on the luminal surface of all blood vessels and is responsible for vital physiological functions of the endothelium. This layer is composed of proteoglycan (PG) core proteins (such as syndecans and glypicans) with a transmembrane domain on the endothelial surface which are crosslinked with highly sulphated glycosaminoglycan (GAG) chains (dermatan sulphate (DS), heparan sulphate (HS), chondroitin sulphate (CS)). Together, these form a thin protective ‘mesh’ on the endothelial surface which incorporates plasma proteins and several biologically active molecules including albumin, superoxide dismutase, xanthine oxidoreductase, lipoprotein lipase, cytokines, endogenous heparins and regulators of coagulation pathway [66]. In I/R, the GCX layer is disintegrated through enzymatic cleavage of of the PG core proteins and GAG chains or direct oxidative stress by ROS [67].

The complex mesh formed by the GCX is an important determinant of vascular permeability which acts as a size-selective and charge-selective molecular sieve allowing small solutes (<70kDa) and water to pass but repels negatively charged plasma proteins to the centre of the lumen. It is now widely accepted that the GCX is primarily responsible for the all-important semi-permeability function of the endothelium [68]. The classical Starling principle of transvascular exchange has been revised to consider the presence of the GCX and more accurately describe fluid exchange in the microcirculation [68]. Disruption of the GCX in inflammatory states leads to vascular hyperpermeability and oedema [69].

The endothelial GCX acts, amongst other sensors, as a mechanotransductor to transmit flow-induced shear forces to the endothelial cytoskeleton, triggering NO production which plays an important protective role in the microcirculatory function in I/R injury. As well as a major determinant of vascular tone, NO has several endothelial protective functions through inhibiting intracellular calcium increase in platelets thereby preventing activation and thrombosis, inhibiting monocyte proliferation and the transcription of leukocyte-binding adhesion molecules (such as VCAM-1). Defective endothelial mechanosensing results in a reduction in eNOS activity causing decreased NO release and anti-oxidant defence. Liver steatosis is a major risk factor for graft failure in liver transplantation mainly due to microcirculatory disturbances. It has recently been demonstrated that steatotic livers have a poor response to shear stress which is an important function of the GCX. Steatotic livers had a reduced level of eNOS activity and NO production as well as decreased Kruppel-like factor 2 (KLF2) expression under subnormothermic perfusion [70]. In a recent study of steatotic rat liver preservation it was shown that IGL-1 preservation solution produced less GCX injury over HTK which was associated with significantly reduced hepatocyte damage and higher NO production after 24 hours of static cold storage [71]. Therapeutic strategies in GCX protection during organ preservation could potenially enhance organ viability of marginal donors and reduce the severity of I/R injury.

The GCX also provides vascular protection by ‘shielding’ the endothelial cells from interaction with circulating platelets and leukocytes – the so called ‘immune camouflage’ effect. The projection of cell surface adhesion molecules such as selectins (PECAM, VCAM), ICAM and integrins (CD11/CD18) are physically shorter than the thickness of a healthy GCX in vessels [72], which inhibits firm adhesion of platelets and leukocytes. Reduced platelet and leukocyte adhesion has been demonstrated with GCX protection in I/R models of isolated guinea pig hearts [73, 74]. Furthermore, degradation of the GCX in cultured endothelial cells under flow has been shown to induce a pro-inflammatory phenotype and increase leukocyte adhesion [75]. Shedding of the GCX layer leads to a vicious cycle of inflammation in two ways. First, a reduction in GCX thickness leaves cell surface adhesion molecules exposed to

circulating inflammatory cells and platelets.which may further facilitate ligand-receptor interactions leading to release of cytokines, matrix metalloproteases and ROS which cause further GCX shedding [76]. Secondly, the inflammatory response can be exacerbated by the circulating degraded fragments of the GCX which have been characterised as DAMPs resulting in cytokine release when binding to TLR receptors on macrophages, dendritic cells and endothelial cells [77] (Figure 1).

There is significant rise in GCX shed products at reperfusion [78] during liver transplantation. It is not clear whether the substantial rise in the recipient circulating GCX products is due to flushing of the shed GCX from the donor liver that has already occurred in preservation or secondary to GCX shedding in the recipient due to reperfusion injury. Given its vital physiological functions, GCX damage could be responsible for several post transplant complications. A recent large clinical study of patients admitted to the intensive care unit with sepsis has shown that GCX shedding is more severe in patients with ARDS and multiple organ failure [79]. Significant GCX disruption in patients with haemorrhagic shock secondary to trauma is associated with the development of coagulopathy and increased mortality [80]. Prevention of local and systemic GCX disruption could potentially provide a novel unifying target to markedly reduce many of the direct and remote complications that arise as a result of I/R injury in the recipient, as well as provide opportunities for donor organ resuscitation during organ preservation.

4. The central role of OPS in organ preservation

In reality, the choice of OPS, and science underpinning their development, are central to organ preservation, whether HMP or SCS are to be used [81]. Ischaemia comes at a huge cost to all cells with aerobic metabolism resulting in cellular injury via a complex and interconnected chain of numerous failing mechanisms essential for homeostasis culminating in cell death. Although cooling suppresses the metabolic rate initially, prolonged cold ischaemia leads to depletion of cellular ATP and accelerated glycolysis as well as lactic acid production. The accumulation of adenine breakdown products such as hypoxanthine initiate oxygen free radical production. Cellular acidosis interrupts pH and energy dependant cellular processes including transmembrane ion pumps (Na/K^+ and Ca^{2+}) which ultimately lead to influx of ions (Na^+ and Cl^-) and water causing loss of membrane potential and progressive cell swelling. Cell membrane injury occurs as a result of lysosomal enzymes release in response to intracellular acidosis and direct oxidative damage from free radicals. The activation of harmful proteases and phospholipidases caused by increasing levels of intracellular Ca^{2+} due to membrane pump failure results in mitochondrial membrane injury with the appearance of mitochondrial permeability transition pores and the release of

Cytochrome C initiating cell death by apoptosis. The combined effect of increase in intracellular acidosis, activation of lysosomes, increasing levels of free Ca^{2+} and Fe^{2+} pools and mitochondrial dysfunction contribute to a pro-oxidant environment which fuel reactive oxygen species production and oxidative stress at early reperfusion. This basic understanding of mechanisms of cell injury in cold preservation underpins the biochemical properties of preservation solutions which aim to target putative pathways mitigating the processes that lead to cell death. Hence, the constituents of OPS include impermeants and colloids to counteract water and electrolyte movement across cell membrane and prevent cell swelling, buffers to control changes in pH, antioxidants in the form of free radical scavengers as well as nutrient precursors for ATP production and energy supply.

Early developments in OPS by Collins et al. were based on impermeants with an 'intracellular ion balance' through reversal of Na^+/K^+ ratios found in plasma. This was later refined with the addition of glucose as an impermeant and removal of magnesium in the Collins C2 solution. The University of Winsconsin solution (UW) has a similar intracellular ion balance with the added benefit of a colloid (hydroxyethyl starch) and pharmacological agents such as allopurinol (xanthine oxidase inhibitor), lactobionate and glutathione (free radical scavengers) as well as adenosine, a precursor for ATP production. Institut-Georges-Lopez-1 (IGL-1) solution is based largely on UW, however, has an ionic balance close to the extracellular ratios of Na^+/K^+ and a less viscous colloid (polyethylene glycol). The Celsior® solution also has an extracellular ion balance and contains mannitol as well as histidine and reduced glutathione. Histidine-tryptophan-ketoglutarate (HTK) or Custodiol® includes histidine and mannitol as impermeants with tryptophan and ketoglutarate as free radical scavengers and nutrient precursor respectively. The Ti-Protec solution, is based on the HTK formulation with a higher fractional ion content, a-ketoglutarate and aspartate as metabolic intermediates, and iron chelators to prevent iron-catalyzed oxidative stress.

Over the last decade, there has not been a great change in the composition of OPS. With the developments in the field of machine perfusion and organ resuscitation to improve the viability of marginal organs there is a greater need for the development of new OPS.

5. New areas of focus in OPS

5.1. Mitochondria-targeted antioxidants

Cold preservative solutions, described in previous review [82], namely Euro-Collins, UW (Viaspan®), Celsior®, Custodiol®, IGL-1®, have been widely used in both experimental and clinical transplantation. Each of them has positive and negative features, and provides similar storage time of donor organs [19, 83]. There is no consensus about the most optimal

preservation solution up to now. To extend organ preservation time and maximise the yield of successful transplantations by improving the quality and function of organ, composition of preservation solutions should be further optimised. A promising approach is inclusion of agents in preservation solutions with targeted mechanisms of action.

Ischemia reperfusion injury (IRI) is largely triggered by mechanisms that involve oxidative stress accompanied by accumulation of reactive oxygen species (ROS). Mitochondria are considered one of the main producers of ROS during cold storage and reperfusion and correspondently, play a key role in IRI of organs. Hence, supplementation of preservation solutions with antioxidants directed at the mitochondria to prevent their dysfunction should minimise organ damage.

Antioxidants are common components of cold preservative solutions. In UW and IGL-1 antioxidant activity is associated with a combination of allopurinol and glutathione. In IRI allopurinol acts as an inhibitor of xanthine oxidase, suppresses the formation of ROS and prevents mitochondrial membrane damage [84]. Glutathione (GSH), which is also a component of Celsior, is a well known natural antioxidant and cellular redox buffer. Due to its redox properties and participation in cellular redox homeostasis and signaling, GSH is now also considered in therapeutics as a tool for stimuli responsive drug delivery systems [85]. In Custodiol, the protection against oxidative stress is provided by tryptophan, which shows a high scavenging capacity [86]. Mannitol is a component of Celsior, Custodiol and other solutions. Besides its osmotic effect, mannitol also plays an antioxidative role via up-regulating the level of catalase which is otherwise decreased during oxidative stress [87].

Rauen and coauthors [88] have found a specific cold-induced increase in the small intracellular iron pool that is responsible for production of high reactive hydroxyl radicals and iron-oxygen species. Chelation of the iron by deferoxamine and lipophilic membrane-permeable LK-614 protects the myocardium and endothelium against iron-dependent hypothermic injury [89]. The authors have developed TiProtec, a new preservation solution, in which the role of antioxidants refers to iron chelators [90, 91]. TiProtec is the modified Custodiol (HTK) solution, which besides iron chelators (deferoxamine and LK-614) contains higher potassium concentration, amino acids (L-glycine and L-alanine) to inhibit hypoxic injury, arginine to increase nitric oxide supply and N-acetyl-histidine instead of histidine as a buffer. It has been previously shown that replacing histidine with N-acetyl-histidine in the Custodiol improved endothelial function in isolated rat aorta [89]. In several experimental systems TiProtec has demonstrated promising results in preservation of hepatocytes, vascular endothelium and smooth muscle cells after cold storage and a period of warm reperfusion. In a recent study [92] iron chelators ensured protection via inhibition of

mitochondrial permeability transition. Addition of iron chelators to cold preservation solutions resulted in increased oxygen consumption rate and regeneration of ATP as well as preserved attachment ability of isolated rat hepatocytes after returning to normothermic conditions.

The intensity of ROS formation depends on the potential on the inner mitochondrial membrane. Consequently, mitochondrial production of ROS is nonlinearly related to the value of the mitochondrial membrane potential with significant increments at values exceeding 150 mV [93]. High values of the membrane potential are greatly dangerous, especially under conditions associated with oxidative stress. Mild uncoupling of oxidative phosphorylation is an approach to preventing hyperpolarization of the mitochondrial membrane. It has been proposed, that the use of water-soluble uncoupler of oxidative phosphorylation 2,4-dinitrophenol (DNP) as a component of cold preservation solution will make it possible to lower oxidative injury of liver [94]. Indeed, supplementation of sucrose-based cold solution with DNP resulted in decreasing ROS production in isolated livers after cold storage, uncoupler wash-out and following normothermic reperfusion, preventing the inhibition of antioxidant enzyme activities, and improving morphology and bile secretion of the liver. The protective effect of DNP at the mitochondrial level involved a decrease in respiration rate in state 4, increase in respiratory control index, and prevention of ATP depletion. Although the existence of high values of mitochondrial membrane potential during hypothermic storage is not documented, these encouraging results stimulate a search for agents with capacity for selective regulation of ROS production by mitochondria.

One of the current strategies for delivery of mitochondrial antioxidants includes their conjugation to lipophilic cations. Lipophilic cations take advantage of mitochondrial membrane potential to facilitate their selective targeting and accumulation within the mitochondrial matrix. Lipophilic cation triphenylphosphonium (TPP^+) conjugated with derivatives of ubiquinol (MitoQ) or plastoquinone (SkQ) are the most advanced mitochondria-targeted antioxidants [95]. The level of TPP^+-conjugated antioxidants in mitochondria can be more than 1000-fold higher than its extracellular level [96]. Both mitochondria-targeted antioxidants, MitoQ and SkQ, are under intensive research and clinical trials, but they are not FDA-approved drugs so far.

In animal models, MitoQ has demonstrated the therapeutic potential in multiple diseases and pathologies: Alzheimer's disease, hypertension, type I diabetes, heart attack, sepsis, fatty liver disease, etc. [95]. The efficiency of SkQ has also been shown on numerous experimental models including those accompanied by ischemia–reperfusion injuries such as kidney and heart infarction and ischemic stroke [97]. SkQ has been used as a component of

cold preservative solution for rat liver hypothermic storage [98]. Isolated rat livers have been stored for 24 h at 4°C either with or without of 1 μM SkQ followed by reperfusion for 60 min at 37°C. The presence of SkQ in the storage solution significantly decreased production of ROS in the liver during cold storage and reperfusion. The addition of SkQ to the cold preservation solution improved energy production function of the liver demonstrated by increased respiratory control index of mitochondria and ATP levels. SkQ exhibited a positive effect on the liver secretory function and morphology after hypothermic storage as estimated from enhanced bile flow rate during reperfusion and partial recovery of organ architecture, state of liver sinusoids and hepatocytes. These results demonstrate that mitochondria-targeted antioxidants are promising components of preservation solution for correction of IRI of isolated organs during cold storage.

5.2. Bioregulators

Improvement of organ survival by treatment with bioactive molecules which enable regulation and modulation of metabolic pathways is an attractive strategy in organ transplantation. Bioregulators may vary in size and nature. Properties of small molecule bioregulators (CO, H_2S, NO) are under intense study as discussed in section 3.2 and have been reviewed in relation to delivery in OPS [81]. Cytokines, growth factors, proteins and other bioactive molecules can be obtained from different sources such as isolated cells, cellular extracts as well as pharmaceutical products. Preclinical and clinical studies in the past decade have demonstrated the renoprotective properties of mesenchymal stem/stromal cells (MSCs) [99, 100]. MSCs are multipotent stem cells that can be isolated from a variety of adult or fetal stromal tissues and have a multi-lineage differentiation potential and the ability to repair damaged tissues and organs after transplantation. The therapeutic action of MSCs is associated with paracrine effects of secretomes, microvesicles and exosomes, which are involved in the transfer of proteins and miRNA to neighbour cells. In total, MSCs secret a lot of bioregulators such as IDO, TGF-a, TGF-b, prostaglandin E2, HGF, IL-6, IL-10, VEGF, bFGF [101, 102]. These, and other not yet identified bioregultors have diverse actions like modulating the local immune system, enhancing angiogenesis, preventing ROS production and cell apoptosis, as well as stimulating survival, proliferation, and differentiation of resident tissue cells [103].

The implementation of MSC paracrine effects requires physiological conditions for synthesis and secretion of bioregulators. Therefore, for low temperature preservation of organs MSCs-based therapy can be valuable on stages of preconditioning or post cold storage reperfusion. Thus, intratracheal MSCs instillation after 8 h of cold storage (4°C) significantly decreased cold ischemia-induced lung injury [104]. The requirement for a source of viable MSCs is a

limitation and raises the speculation as to the use of their conditioned media or natural mixture of bioregulators from other sources instead of MSCs themselves. It has been shown that supplementation of UW solution with trophic factor mixture (insulin-like growth factors, epidermal growth factors and nerve growth factor, bactenicin and substance P) substantially increased viability and reduced the production of hydrogen peroxide by tubular cells of dog kidneys after 3 days of cold storage [105], protecting mitochondrial function and preventing apoptosis [106].The presence of trophic factors enabled the prolongation of storage duration time (up to 6 days) and significantly improved post-transplant kidney function [107]. The efficiency of storage medium supplemented with such growth factors has been also shown in a pig allogenic liver transplantation model [108].

The effects of bioregulators present in fetal tissue cytosol (FTC) of mesenchymal-mesodermal origin have been studied on a model of rat isolated liver. The choice of this source was based on high percentage of stem and progenitor cells and similarity with microenvironment of MSCs. The unique composition of bioactive molecules and trophic factors of fetal origin has demonstrated a high recovery in activity in several experimental systems [109–111]. It was shown that rat pretreatment with FTC for 4 h before long-term (24 h) liver cold storage followed by reperfusion for 60 min led to a decrease in free radical production and normalisation of antioxidant enzyme activity [112]. The pretreatment also had a positive effect on ATP level and restored the activity of a key ATP-generating enzyme of glycolysis - pyruvate kinase, as well as increasing the level of succinate-dependent mitochondrial respiration after short-term hypothermic storage [111]. Recently, these authors have shown that FTC presence in the sucrose-based cold preservation solution stabilised pro-oxidant-antioxidant balance which is impaired in liver after hypothermic storage for 24 h followed by reperfusion, prevented the uncoupling of mitochondrial oxidative phosphorylation and ATP level decline [113]. In addition to observed biochemical changes, supplementation of cold preservation solution with FTC resulted in almost complete restoration of bile flow rate during reperfusion and normalisation of organ morphology. It is interesting that natural cocktail of trophic factors contained in FTC demonstrated a more powerful protective effect on cold ischemia-induced liver injury than epidermal growth factor and insulin-like growth factor-I added separately or in combination to University of Wisconsin solution [114]. The great protective potential of bioregulators of fetal origin as the component of cold preservation solution enforces identification of individual bioactive molecules and investigations of mechanisms of their action.

6. Clasification of perfusion techniques based on preservation temperatures

In the history of perfusion machines, different strategies and temperatures of perfusion have been cited without consensus between authors. Terms such as hypothermic, subnormothermic or normothermic were frequently used to describe perfusion temperatures, and the absence of a standardised criterion to describe technical details led to great heterogeneity between studies. In 2016, a group of distinguished researchers in the field of liver preservation proposed a nomenclature of perfusion temperature ranges that facilitate the comparison of different studies, meta-analysis realization and criteria homogenization [115]. Much work in this area has focused on liver perfusion, although the same broad concepts apply to other organs.

6.1. Hypothermic machine perfusion (HMP) (0°C-12°C)

Perfusion at this temperature range mainly reduces tissue metabolism and at the same time through the preservation solution provides the necessary metabolic substrates for ATP synthesis and removal of metabolic waste by washing the parenchyma and endothelium [116]. The proposed cut-off point for HMP at 12°C is supported by the observation of numerous energy-dependent reactions of mitochondrial liver enzymes which exhibit significant rate changes at this temperature [115].

Accordingly, some of the benefits of HMP are: minimises cold ischemia injury, improves grafts viability and protects against biliary lesions [117, 118]. In the case of Hypothermic Oxygenated Perfusion (HOPE), it also provides the opportunity to restore mitochondrial redox activity and restore cellular energy status, while the metabolism is still dampened by hypothermia [119]. HMP additionally reduces the inflammatory response preventing Kupffer cells activation and decreasing neutrophils and platelets activity during reperfusion [120] and is considered as a safe technique because, in the case of machine failure, the graft simply returns to the standard Cold Storage (CS) conditions. On the other hand, the main disadvantage of HMP is that the assessment of liver function "in real time" is not feasible with this method. For instance, liver does not synthesize bile during hypothermia [121].

The first experience in humans was reported by Guarrera et al. in 2010 with non-oxygenated hypothermic dual (portal vein and hepatic artery) perfusion of twenty standard DBD human livers. After 12 months of transplantation, the survival rate was 90%, early allograft dysfunction occurred only in 5% of the patients, while the incidence of biliary stricture was 5% [122].

Van Rijn et al [123] tested a dual hypothermic oxygenated MP (DHOPE) over DCD livers. The biliary outcomes were also encouraging; none of the preserved livers required retransplantation for nonanastomotic biliary stricture, as opposed to 5 of 20 livers in the control group. In the largest HMP clinical trial performed, 50 recipients of extended DCD livers achieved superior outcomes compared with nonperfused DCD grafts and perfused DBD grafts [124].

6.2. Midthermic machine perfusion (MMP) (13°C-24°C)

In view of the fact that the broad range of temperatures, between 12°C and 33°C, shows a great difference in the rate of metabolism, Karangwa et al. [115] considered more appropriate to subdivide this interval and proposed the term midthermic machine perfusion.

Midthermic and subnormothermic machine perfusion were proposed to achieve a balance between the deleterious effects of cold ischaemia and the high metabolic requirements of the normothermia [125]. Additionally, an exposure of the graft to normothermic temperatures after a period of CS implicates a significant risk of oxidative stress burst.

The application of midthermic machine perfusion resulted in lower intravascular resistance, a better conserved microcirculation and a stronger mitochondrial function, and these effects coincided with both, a higher energy charge and bile production [121].

Bruinsma et al examined the impact of a 3 h midthermic (21°C) perfusion on 7 discarded livers. The grafts were gradually warmed reaching the final temperature within 1 hour. Liver function and dysfunction parameters showed not only that there was no liver injury, but also significantly improved liver functionality [126].

6.3. Subnormothermic machine perfusion (SNMP) (25°C-34°C)

Graft perfusion between 16-25°C is possible without the presence of red blood cells. It simplifies the procedure and could reduce costs. This is feasible because perfusion at subnormothermic temperature diminishes activity of respiratory chain in mitochondria and decreases the demand of cellular energy [127].

In subnormothermic perfusion of pig livers, the hepatic and biliary injury was reduced in grafts harvested by donation after cardiac death (DCD) and steatotic livers [128]. In addition to the biliary improvements, lower serum levels of alkaline phosphatase were measured during the survival period in the group treated with SNMP, compared with the control group. The improvement of the preservation protocols of marginal grafts has the potential to increase the donor pool and improve graft function after liver transplantation.

Controlled oxygenated rewarming (COR) is the most recent application of the perfusion machine, in which the perfusion begins at hypothermic temperatures and gradually increases to subnormothermia [129], improving restitution of cellular homeostasis and mitigating rewarming injury by adapted increase of temperature and metabolism [120].

COR has recently been clinically applied, with the successful transplantation of six DBD grafts accepted under 'rescue allocation' criteria [130]. The COR group demonstrated a lower AST peak compared with controls and 100% of patients and grafts survival at 6 months.

It is expected that subnormothermic *ex vivo* perfusion machine will have a cooperative role with other preservation techniques [131]. An example of this is the supercooling technique, which was used to preserve rat livers up to 96 h [132]. Supercooling was combined with subnormothermic machine perfusion [131] for the loading of cryoprotectants into the liver as well as postsupercooling rewarming. Prior to supercooling, researchers used subnormothermic perfusion to load isolated rat livers with a nonmetabolizable glucose derivative (3-O-methyl-d-glucose, 3-OMG). Livers were supercooled avoiding intracellular ice formation and, at the end of supercooling, 3 h of subnormothermic perfusion was performed to achieve an adequate recovery period for the liver before orthotopic transplantation. Three-month recipient survival in the group that received livers supercooled for 72 h at -6°C was 100%.

6.4. Normothermic machine perfusion (35°C-38°C)

The term normothermia usually refers to the physiological body temperature of the species used in the study, i.e. 37°C for human and rodent studies and 38°C in studies with porcine models. The idea underlying the technique is to replicate the normal metabolism of the liver outside the body, providing oxygen and essential substrates in an environment maintained at a normal temperature (37°C), avoiding ischemia and hypothermia altogether [121]. One of the main advantages of normothermic machine perfusion is the opportunity to evaluate the viability of the organ before its transplantation [120] by measuring markers of hepatic metabolism (i.e. bile production and liver enzymes release). The difficulty lies in providing sufficient oxygen and other necessary substrates to prevent subsequent graft deterioration and bacterial contamination which is more likely to occur in warm environments [125]. Typically, perfusates based on concentrates of red blood cells, plasma, nutrients, cofactors and insulin, antibiotics, electrolytes and buffers are required, which makes normothermic machine perfusion a complex and expensive procedure [126]. To avoid the use of human blood products, the interest in extracellular oxygen carriers (EOC) or oxygen-carrying plasma expanders (OCPE) increased [133].

Normothermic machine perfusion technology was initially developed to mitigate the negative effects of simple CS, but additional benefits have allowed its use for broader purposes [134]. Normothermic perfusion defatting seeks to augment the protective effect of perfusion by adding a pharmacologic 'defatting cocktail,' stimulating lipid metabolism. Use of a defatting cocktail reduced intracellular lipid content by 50% during 3 h of normothermic machine perfusion in steatotic rat livers [135]. Another additional benefit is the ability to extend the graft preservation times. In the first clinical series, Ravikumar et al. [136] demonstrated feasibility of preservation over 20 h without compromising recipient outcome. Liu et al. [137] recently reported a case describing the metabolic activity of a discarded human liver perfused for 86 h on an *ex situ* normothermic machine perfusion. This is an important finding, since long-term preservation could provide an opportunity for tissue regeneration and organ reconditioning through pharmacological, immunological and genetic interventions. As a demonstration of intact metabolism, bile production and lactate clearance were preserved until the end of 86 h. The hepatic histology of the parenchyma showed completely healthy hepatocytes. One limitation of the work, worthy of mention, was the absence of a transplant.

Nasralla et al. described the first multicenter randomized clinical trial in which normothermic perfusion was compared with static CS. 170 livers were perfused in a normothermic machine and 164 preserved by static CS, and the authors reported 1-year outcome for those successfully transplanted. There was a significant reduction in graft injury despite a 50% lower rate of organ rejection and a 54% longer mean preservation time. There was also no advantage to be gained from using normothermic perfusion in terms of any major outcome measurement, such as graft or patient survival, or frequency of biliary complications [138]. Mergental et al. reported liver transplants after a period of normothermic perfusion to assess the viability of the rejected livers. They used these livers after a variable period of CS and reported encouraging results, with an average hospital stay of 10 days and graft survival in all recipients [139].

It is probable that in the proximal future, the normothermic perfusion will be used to change the concept of preservation, to a concept of being a method of treatment and repair of organs [140].

7. Experimental observations of temperature on liver preservation perfusion protocols.

Liver hypothermic/midthermic/subnormothermic perfusion (**H/M/S**) effectiveness will depend on the following fundamental features:

1) Counteract cellular edema due to hypothermia: this phenomenon could be compensated by the inclusion of a cell impermeant substance into the perfusion solution [4].

2) Prevent the expansion of the interstitial space and the subsequent compression of the vasculature that, during reperfusion, will generate an increase in intrahepatic resistance and vascular damage. With the inclusion of an oncotic active substance in the perfusion solution, it could be possible to control the water distribution between interstitial and vasculature during perfusion [141].

3) Provide adequate conditions for high-energy phosphate regeneration, therefore it will be possible to improve the function and viability of the liver during reperfusion. Accordingly, the addition of nucleotides and other substances into the perfusion solution could improve the organ function and viability after the preservation procedure.

4) Supply an adequate gas atmosphere to obtain a lower oxidative metabolism preventing the toxic effects of high oxygen concentrations in the perfusion solution [142].

Specifically, colloid osmotic pressure (COP) or plasma oncotic pressure represents the counterforce to the filtration pressure in the blood circulation and it is therefore, partially responsible for the water distribution between the vasculature and the interstitial space [143]. Its physiological value is approximately 25 mmHg and it was determined mainly by Albumin concentration (Alb) in the blood. For long-term perfusion experiments, oncotically active substances are essential to counteract interstitial edema, inevitably produced when these substances are not added to the perfusion solution.

COP could have effects on:

- Variables such as the total H_2O content of tissues and their distribution in intracellular and interstitial spaces during hypothermic perfusion.

- Lower hydrostatic pressure gradient developed during the **H/M/S** perfusion procedures.

- The same variables in the post perfusion and their effects on flow, intrahepatic resistance, tissue damage and bile production.

It is possible to ask: which is the optimal COP of a solution for **H/M/S** perfusion procedures? Since it is necessary to maintain an appropriate intravascular fluid level in presence of a

lower perfusion pressure than the physiological it is important to consider the inclusion of a colloid in the hypothermic perfusion solution, for which the following experiments were carried out.

The COP (expressed in mmHg) was determined using a colloidal osmometer or oncometer at 20°C (Table 1). The optimal condition would have been the determination of the COP at low temperature, but this is not possible yet, because commercial equipment is not available. However, a temperature correction could be made by using an appropriate formula if one were available.

COP correction by temperature

Nearly all publications indicate that the COP was determined at room temperature (20-25°C). Skillman [144, 145] corrected this values at 37°C, based in the work of Soto-Rivera [146], who used the Hepp osmometer to determine COP for comparison against the plasma density.

The van´t Hoff estimation for osmotic pressure is:

$$\pi = CRT$$

where C is the molar solute concentration, R is the ideal gas constant and T is the absolute temperature (Kelvin). Then the effect of the variable temperature could be calculate as:

$$\pi_{calculated} = \pi_{measured} [(273°C + \text{calculated temp. °C}) \div (273°C + \text{measured temp. °C})]$$

in which $\pi_{calculate}$ and $\pi_{measured}$ represent the calculated and measured values respectively, for the π at their corresponding temperatures at °C.

If a COP determined at 20°C is 11.3 mmHg, then the value at 5°C will be calculated as:

$$\pi_{calculated} = 11.3 \text{ mmHg} [278 \div 293] = 11.3 \text{ mmHg} \times 0.9488 = 10.72 \text{ mmHg}$$

In the following example, determinations were done by using an OSMOMAT 050 oncometer from GONOTEC with a semi-permeable membrane of 50000 Daltons cut-off.

Our group have been interested in developing a new group of OPS formulated on gluconate and the Good's buffer BES (N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid) with different colloids (e.g. BES gluconate HES-BG-HES [147]). Their physico-chemical properties have been studied and compared with other traditional OPS [81]. Experimental COP values obtained for the newly formulated gluconate-based BG-PEG and BG-HES preservation solutions [147] were determined at different concentrations of two different

colloids: PEG 35000 and hydroxyethyl starch (HES) used at 1, 2, 5, 10 and 20 g/L at 20°C. The results are presented in Figure 2.

At similar colloid concentration the COP developed by the PEG 35000 is higher than that of HES. In fact, using the obtained equations, it is possible to calculate the colloid concentration and obtain an appropriate COP in the perfusion solution.

Theoretical calculations to determine the appropriate concentration of colloid substances that produces a COP suitable for a H/M/S perfusion solution.

Based on the classical Starling´s equation the purpose of this study was to obtain an accurate and simple method for calculating the COP concentration to apply in an H/S protocol. We based this calculation on the graphic shown in Fig 2. These graphs were obtained plotting the COP measured at different concentrations of oncotic substances like as PEG 35000 and HES in BG- basic perfusion/preservation solutions at 20°C.

The classical Starling hypothesis [148] can be expressed as:

$$F = K_f [(P_c - P_{isf}) - (\pi_{pl} - \pi_{isf})]$$

where F is the capillary filtration; K_f is the membrane permeability component; P is the hydrostatic pressure; π is the colloid osmotic pressure, c is capillary, pl is plasma, and isf is interstitial fluid. As was reported by Weisberg [143] the systemic capillary forces could be represented in a simplified frame, as it is shown in Table 3.

If we consider a hypothermic perfusion protocol in which the perfusion pressure is 25% lower (4.2 mmHg) than the physiologic one, the calculated outward flow will be 15.3 mmHg (see Hypot. Perf. Protocol in the frame). Then we should provide an oncotic substance that in the perfusion solution achieves a COP of 15.0 mmHg.

The equation corresponding to PEG 35000 added into BG-basic solution showed in Figure 2 is:

$$Y = 4.101\ e^{(0.0889\ x)}$$

where Y = COP (mmHg) and x is the colloid concentration in g/L

Solving the equation, we obtain a PEG 35000 concentration of 14.81 g/L necessary to develop a COP of 15 mmHg at 20°C.

At 5°C the value of calculated COP will be 14.40 mmHg (see above), then will be necessary to make a correction of PEG concentration (4%), adding 0.59 g/L of PEG obtaining a final concentration of 15.40 g/L.

In order to compare BGP-HMP with other traditional preservation solutions [81], such as HTK [81, 149], BGP-CS [149] and Viaspan (UW) [4, 81] we determined different physicochemical parameters: COP (mmHg), osmolality (mOsm/Kg H_2O) and viscosity (cP) of the solutions (see Table 1). Solution compositions are shown in Table 2.

Using theoretical calculations expressed above, it is possible to determine the appropriate PEG or HES concentration which produces a suitable COP for hypothermic protocols.

Although hypothermic perfusion protocols are designed to recreate a physiological environment, this model is quite far from the normal physiology of an organ. For example, hypothermic perfusion differs from physiological perfusion in the following points:

A) The perfusion pressure is only 25% of the normal pressure (40 mmH_2O vs 180 mmH_2O)[150].

B) The procedure of hypothermic perfusion is maintained at a temperature of 5°C.

C) At that temperature, the consumption of O_2 by the tissue is approximately 5% of the physiological requirement at 37°C [151].

Point C, involves a controversy not yet resolved, which is the appropriate oxygen concentration in a **H/M/S** perfusion solution?. It will be necessary to establish the oxygen consumption by the organ with the perfusion protocol used to assure the correct gas concentration at the flow and pressure utilized. This research involves:

i. The oxygen solubility in the perfusion solution at the perfusion temperature.

ii. The organ oxygen consumption at the perfusion temperature.

Solubility curves of O_2 at different temperatures in BGP–HMP (Figure 3A) and HKT (Figure 3B) solutions were experimentally determined as it was previously reported [152].

In previous experiments, a lower oxygen consumption from hypothermically perfused rat livers was reported [151]. The O_2 consumption of isolated rat livers perfused at 5 and 10°C with BGP–HMP or HTK solutions saturated with air were also established [153]. It is necessary to provide appropriate oxygenation of the preservation solution during HMP perfusion protocols, as a requirement for effective preservation and maintenance of organ viability and function.

8. Experimental observations about the viscosity (η) of the perfusion solutions at low temperatures

Viscosity is a measure of the resistance of flow due to internal friction of a fluid. Owing to viscosity, it is necessary to apply a force when one layer of fluid is caused to move in relationship to another layer.

When the internal flow resistance is independent of the external force, as is the case with water, the fluid is called a Newtonian fluid. In the case of non-Newtonian fluids, they change viscosity when exposed to different shearing stress, and viscosities of such fluids are called apparent viscosities.

The temperature strongly influences the fluid´s viscosity. The viscosity of blood at 4°C is double that at 37°C [154]. In the liver, vascular resistance correlated well with the viscosity of the perfused fluid. In addition, the concentration of the oncotic agent contained in the preservation solution strongly affects its viscosity. It was shown that in static preservation procedures, the viscosity of the initial flushing solution may play an important role in determining the outcome of organ procurement from non–heart beating donors [155]. It was reported that HES in UW preservation solution affects the organ washout due to red blood cell aggregation [154]. Livers flushed with solutions of low viscosity showed lower vascular resistance than those flushed with cold UW, and improved the viability of the organ.

When considering preservation perfusion procedures such as hypothermic/subnormothermic perfusion, the effects of temperature on fluid viscosity have not been rigorously investigated. For these reasons, it is important to study the results of this combination of effects on the solution viscosity and how this affects the organ outcome after preservation. To achieve this, the experimental results of different temperatures on viscosity of BG-basic preservation solution at different PEG concentrations are needed.

The BG-basic solution has the same chemical composition to that of BGP-HMP solution without oncotic agents (Fig 4). We also determined the effect of adding HES to the BG-basic solution at different temperatures (Fig 5). Experimental results were obtained with a rotational digital Viscosimeter (LCD Shanghai Nirun Intelligent Technology, model SNB-2).

The BGP-HMP solution was developed for hypothermic perfusion [147] and also tested for static preservation [149], the oncotic agent used was PEG (polyethylene glycol 35000) at a

concentration of 1 g/L. Fig 4 shows the effect of temperature on BG-basic solution viscosity at different PEG concentrations. A decrease of temperature exponentially increases the viscosity of the solution; this effect is magnified by the raise in PEG concentration. Interestingly, the substitution of PEG by HES increases the viscosity of the preservation solution with a temperature reduction, but this increase in concentration does not affect the viscosity in the same way as PEG. Finally, we compared similar concentrations of both compounds (PEG and HES) at low temperatures; PEG 35000 viscosity was significantly higher than HES viscosity.

Figure 4 shows the comparison of the effect of temperature on the viscosity of the preservation solutions UW, HTK, BGP-HMP, BGP 35 and water.

In CS preservation, the role of the preservation solution viscosity becomes essential during organ washout to enable its distribution through the vasculature of the entire organ, preventing the effects of cold ischemia. But, in hypothermic/subnormothermic perfusion techniques, where long perfusion times and good performances of the organ vasculature are required, the viscosity of the perfusion solution is associated with the concentration of the oncotic agent in a delicate balance between the control of interstitial edema and the perfusion flow and vascular resistance.

9. Analyzing perfusion parameters in the preservation of organs

New technologies that are being developed in organ perfusion, which involve new roller pumps, new blood gas exchange devices, new centrifugal heads, miniaturized devices, and of course, the development of the perfusion training programs mainly during the past 20 years have led to the scientific evolution of perfusionists and to the development of these techniques. Ultimately, these new technologies are trying to create an appropriate environment to preserve the organ in the best possible way, outside of its usual environment, and maintain this situation until the organ is implanted. However, many debates still remain, dealing with the ways of achieving the optimal perfusion, but mainly with the relationship between perfusion pressures and the optimal preservation solution flow during extracorporeal circulation [156]. For instance, roller pumps by their design produce a pulsatile wave pattern of flow, which by producing the appropriate pulsatile wave could serve to overcome the opening pressure for the capillary bed, sufficient to allow brief, but ongoing ‘bursts’ of perfusion, whilst avoiding continuous exposure to this higher pressure which is recognized as a contributing factor to develop a deleterious tissue edema [142].

Improvements in organ preservation solutions continue to be made with new pharmacological approaches. New solutions have been developed for dynamic perfusion

preservation and are now in clinical protocols [81]. The basis of this hypothermic protection is that cooling can help to combat the deleterious effects of ischemia, but the consequences of cooling are not exclusively beneficial. Hypothermic storage is a compromise between the benefits and detriments of cooling [157]. Preservation advances allow the use of perfusion circuits to mimic healthy physiological conditions, which it was originally presented by Belzer in 1973 [158]. This was based on the hypothesis that organs are best preserved in a condition as physiological as possible, by providing oxygen together with sufficient nutrients to support a constant metabolism. Initially this preservation was conducted at body temperature. Only later, the development of hypothermia and Single Cold Storage (SCS) was introduced, where the organ was cooled in vivo through a flush out with a cold preservation solution and subsequently stored on ice [159]. Inside this paper, Vekemans et al. has compiled an interesting list of references related to pressure, flows, species, and temperature during hypothermic perfusion or normothermic machine perfusion. These actions permit the organ to recover from cellular stress and tissue injury during donor death and organ procurement, which can contribute to organ injury and rejection following transplantation, and also enable therapeutic intervention before transplantation. The advent of *ex vivo* organ perfusion shows promise to make larger pools of donor organs available by enabling rehabilitation of organs that would otherwise be unsuitable for transplantation [160].

The other potential risk for organs exposed to continuous oxygenation at low temperatures during hypothermic perfusion is production of oxygen-derived free radicals (OFR) with consequent cell damage, either because abnormally high oxygen tensions may be used by deliberate oxygenation of the perfusate, or because the normal antioxidant defenses, depending on consumption and regeneration of antioxidants, are disrupted at low temperatures [142].

9.1. Warm perfusion

If one accepts the overall premise that perfusion of organs is a valuable approach to maintaining function, the obvious question is: why use low temperatures? Why not perfuse at normal body temperatures and avoid the metabolic challenge of cooling?

From the preceding sections it will have become apparent that normothermic conditions were applied early in the history of *in vitro* organ perfusion, but the challenges of providing good oxygen carrying (using blood or blood components) without encountering problems of thrombus formation, vascular damage and infection were insurmountable with the technology available at the time. A major concern when applying warm perfusion has been to supply not only sufficient oxygen-carrying capacity to support aerobic metabolism, but in addition all the other physiological mediators (the various substrates and co-factors) which are required for

regulated homeostasis in the isolated organ. In some ways this can be considered as analogous to the complex media developed for cell culture *in vitro*. Viewed from a different perspective, no advantage would be achieved from using a warm perfusion system, which induced early signalling markers for stress or inflammatory processes. The various components of normal blood provide the ideal perfusate for warm perfusion, however requires sophisticated technology to maintain oxygenation and adequate intravascular flow, without inducing activation of circulating blood cells, micro thrombi and associated negative effects on organ microcirculation (a situation in some ways analogous to cardiopulmonary bypass). To replace blood with a cell-free synthetic solution poses different, but equally difficult questions about reproducing the complete range of blood functions [142].

10. Summary and Future Horizons

Almost a decade has passed since our UNESCO Chair grouping reviewed how organ preservation was then being practiced, and what research areas were potentially signposting new possibilities for clinical translation [82]. Organ perfusion was just re-establishing itself as a valuable clinical option for *ex vivo* preservation, after many years in which it had not been considered mainstream. Now, dynamic intervention by continuous perfusion is one of the most-discussed topics at transplantation meetings, with different systems and ranges of temperatures being applied in trials in all the major organ classes. These in turn are raising exciting possibilities for organ conditioning or repair as novel therapies during the perfusion period, which may well become a ‘hot topic’ for research over the next ten years. The concept of true long-term organ preservation at deep sub-zero temperatures also seemed to be at an impasse in previous times [82], whereas now there are small signs of renewed interest and investigation [161–163], although these currently remain far from clinical application. In turn, these will depend on new kinds of dynamic perfusion of some kind. Perhaps these will be accelerated towards translation by new ideas and better understanding of the underpinning scientific principles by studies in cryobiology. Whichever technologies become predominant, the times ahead will certainly be very interesting in the quest to develop better organ preservation strategies and understand the cell and molecular changes in organs outside the body, which form the ‘supply line’ for clinical transplantation.

Aknowledgements

The authors would like to acknowledge the importance of the UNESCO Chair in Cryobiology in the formulation of this review for providing the international contacts and stimulating collaborative research between our groups in different parts of the world. Part of this work was supported by the Italian Liver Foundation via a grant of the International Relationship Program of Regione FVG, Italy. AP and AS acknowledge support of the Institute for

Problems of Cryobiology & Cryomedicine, Kharkov, Ukraine, and Kharkov State University. We are also grateful for the Heptaobiliary Surgery Fellowship research funding by The Wellington HCA Hospital (London) for Mr Farid Froghi.

Figures and legends

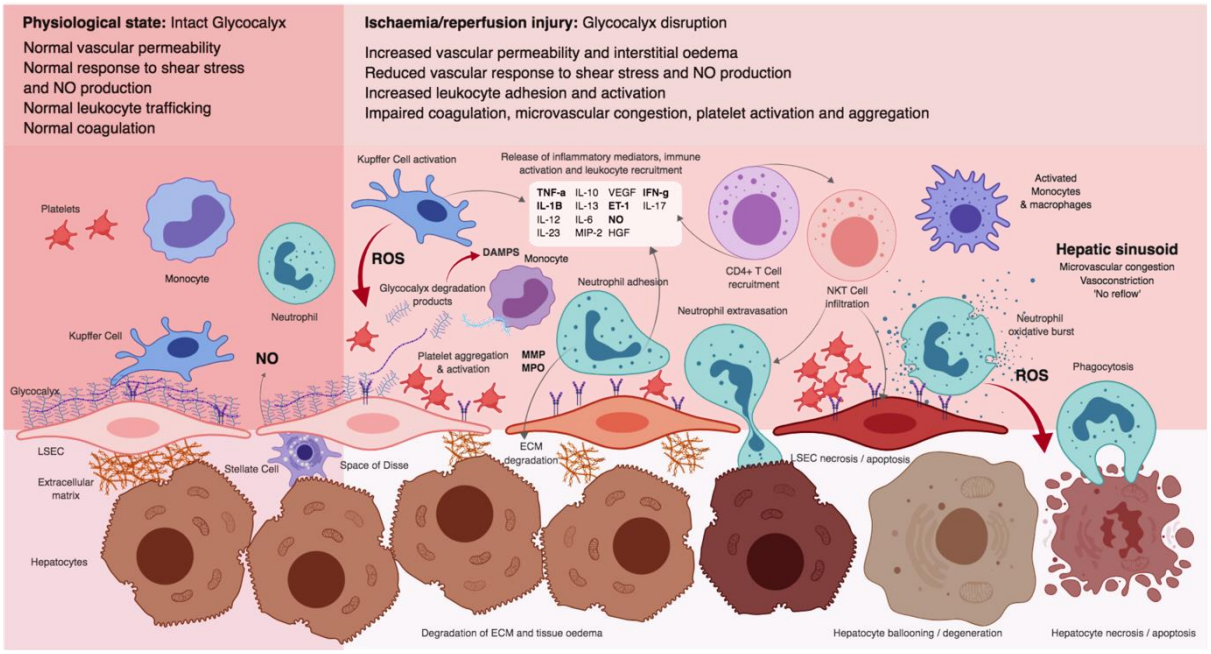


Figure 1. In a physiological state, the healthy glycocalyx maintains normal vascular permeability, response to shear stress and NO production. Early events in I/R injury include activation of Kupffer cells and ROS and cytokine production which cause direct or indirect damage to the endothelial glycocalyx in the hepatic sinusoid. This results in increased platelet and leukocyte adhesion, as well as a loss of vascular permeability leading to tissue oedema. The glycocalyx degradation products act as DAMPs further exacerbating the inflammatory response with the release of cytokines and recruitment of inflammatory cells (neutrophils, macrophages, CD4+ and NK T cells) to the liver post reperfusion. The combined effects of tissue oedema, microvascular congestion from platelet and leukocyte aggregation and direct effect of inflammatory cells lead to liver sinosiudal and hepatocyte cell death.

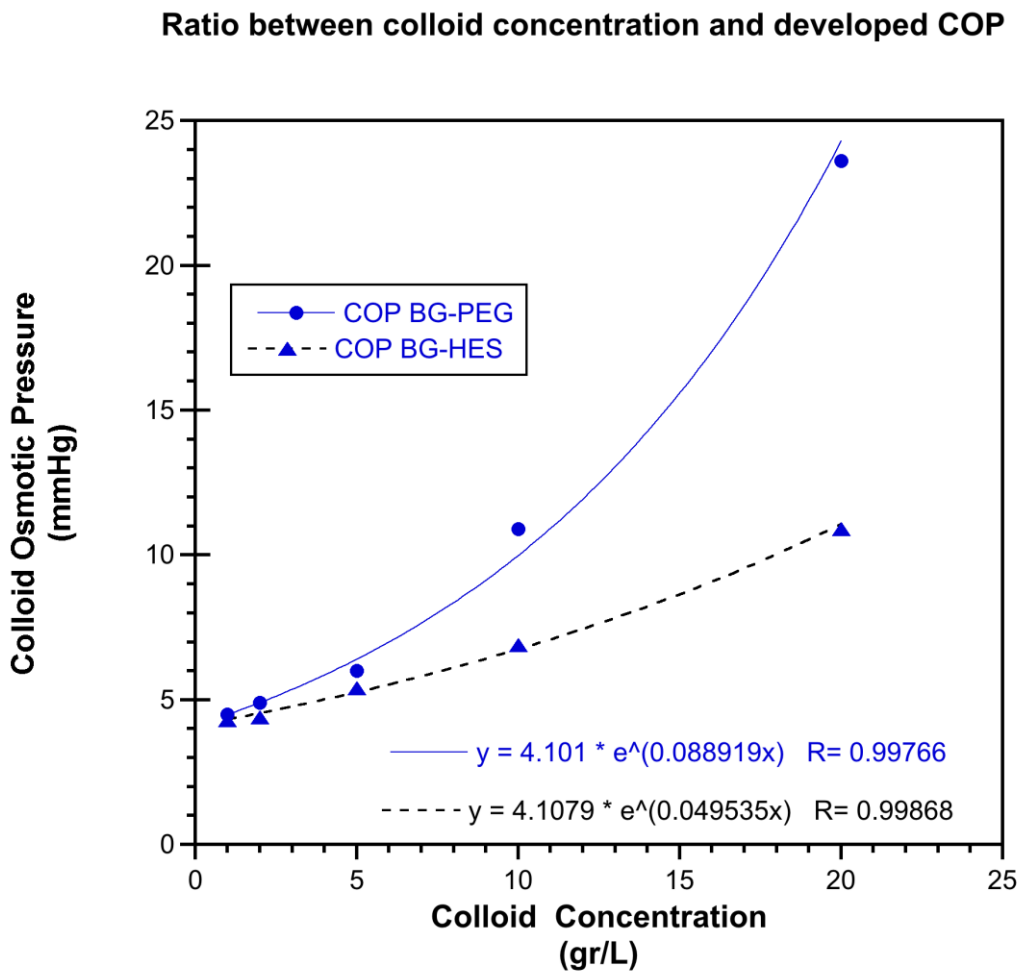
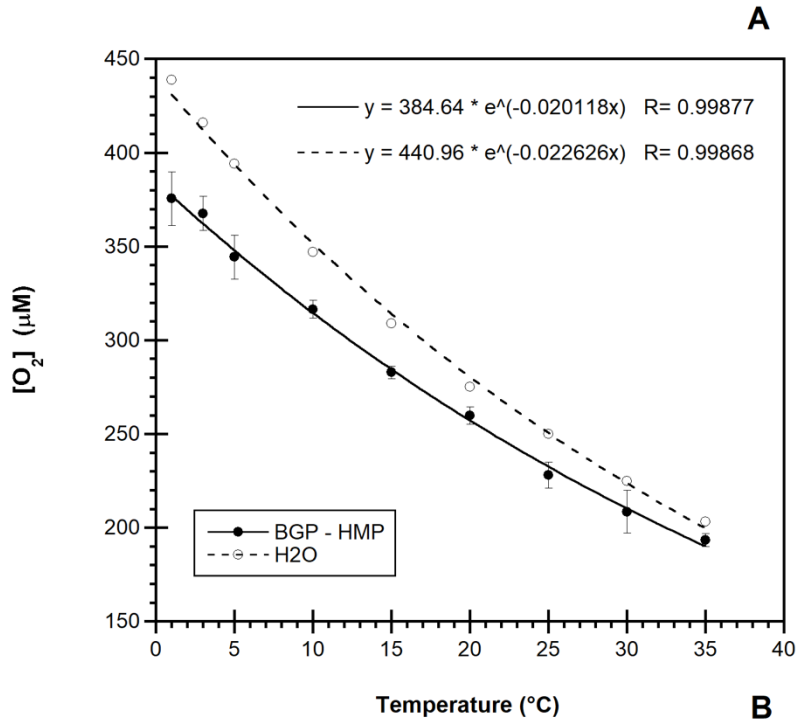


Figure 2. Effect of PEG and HES concentration on BG-basic solution COP. Higher concentrations of PEG result in a major COP than the observed for the same HES concentration. Data were fitted by an exponential equation.


A
y = 384.64 * e^(-0.020118x) R= 0.99877
y = 440.96 * e^(-0.022626x) R= 0.99868
BGP - HMP
H2O
[O2] (µM)
Temperature (°C)

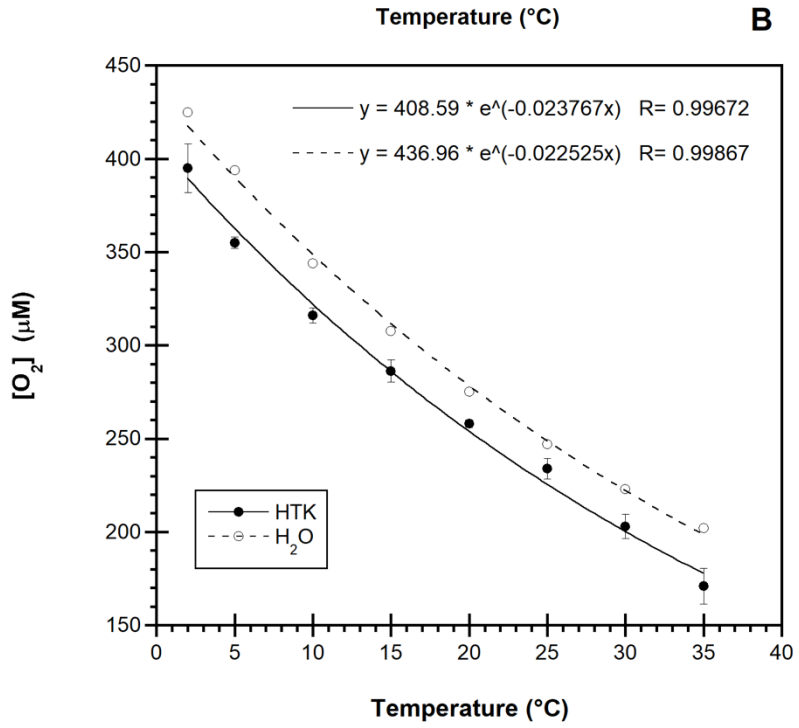

B
y = 408.59 * e^(-0.023767x) R= 0.99672
y = 436.96 * e^(-0.022525x) R= 0.99867
HTK
H2O
[O2] (µM)
Temperature (°C)

Figure 3. Effect of temperature on oxygen carrying capacity of BGP-HMP and HTK solutions respect to water. Oxygen solubility in fresh water and (A) BGP-HMP solution or (B) HTK solution.

Oxygen solubility values in pure water were taken from bibliography and a barometric pressure of 754.4 mmHg was employed for calculations. Oxygen concentration was calculated: Ln $[O_2]$ = $-\Delta H / RT + C'$, being: $[O_2]$ the oxygen concentration at a given temperature; T the thermodynamic temperature; R the gas constant (8.314 J /K.mol); ΔH the heat of solution in kJ/mol and C' a constant. An extrapolation was used for calculating the oxygen concentration at different temperatures, as in the following example: the HTK oxygen solubility at 5ºC and 760 mmHg of barometric pressure was 370 µM O_2, which, as expected, is lower than the oxygen solubility in fresh water in the same conditions (397 µM O_2). Values are represented as the mean±SD of 5 measurements.

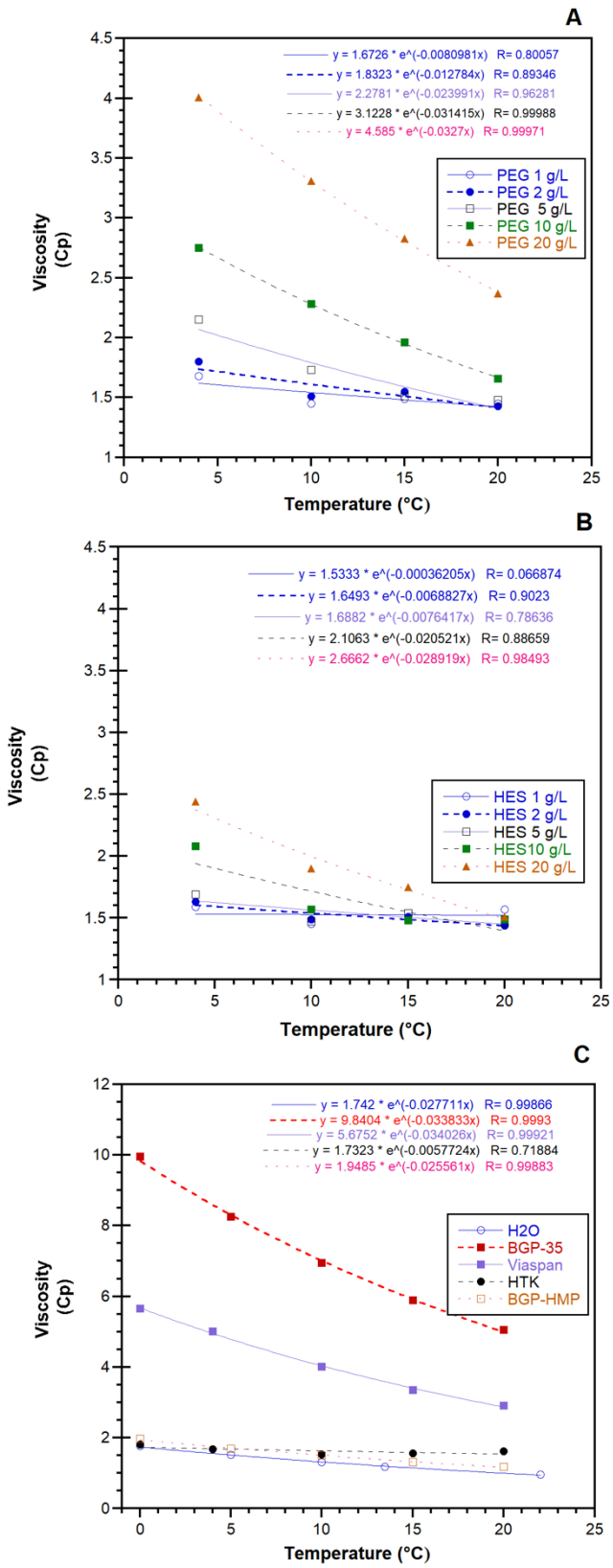


Figure 4. Effect of temperature on preservation solutions viscosity. (A) Viscosity of BG-basic solution at different PEG concentrations. (B) Viscosity of BG-basic solution at different HES

concentrations. (C) Effect of temperature on preservation solutions viscosity respect to fresh water. Viaspan (UW solution), HTK (Bretschneider solution), BGP-HMP (Bes-Gluconate-Polyethylene glycol for Hypothermic machine perfusion solution), BGP-35 (Bes-Gluconate-Polyethylene glycol (40 g/L) for liver microorgans cold storage solution [164]). All data obtained were fitted by an exponential equation showing a good regression coefficient (equations are inserted in the graph). The displayed curves show clearly the increase of viscosity due to the reduction of temperature in all studied solutions.

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Table 1: Determinations of COP, Osmolality and viscosity of different preservation solutions. (n=number of measures).

Preservation solution	HTK	Viaspan (UW)	BGP-CS	BGP-HMP
COP (mmHg) 5°C (n=4)	1.45 ± 0.46	31.90 ± 1.63	11.89 ± 1.78	4.35 ± 0.24
OSM (mOsm/Kg H_2O)	310	324	303	290
Viscosity (cP) 5°C (n=3)	1.68 ±0.005	5.01 ±0.007	2.61 ± 0.005	1.68 ± 0.010

Table 2: Preservation solution composition

Preservation solution	**UW** [81]	**HTK-Ti-Protec** [81]	**BGP-HMP [165]**	**BGP-CS**	**BG-basic**
Trasplantation era	1990s-current	Future-in evaluation	Future-in evaluation	Future-in evaluation	Future-in evaluation
Electrolytes					
Cations (mM)					
Na^+	30	16	100	100	100
K^+	125	93	7	7	7
Mg^{++}	5	6	5	5	5
Ca^{++}		0.05			
Anions (mM)					
Cl^-		103			
HCO_3^-					
PO_4^- (a)	25		2.5	2.5	2.5
SO_4^{--}	5		5	5	5
Lactobionate$^-$ (b)	100				
Buffers (mM)					
Histidine		198			
Glycine		5	15	15	15
Tryptophan		2			
BES			30	30	30
Impermeants (mM)					
Glucose		10			
Raffinose	30				
Sucrose		37	20	20	20
Colloids					
HES (g/L)	50				(*)
PEG 35000 (g/L)			1	11	(**)
Osmolality	320	305	290	303	290
pH	7.4	7.0	7.4	7.4	7.4

Pharmacological agents					
Adenosine	5		5	5	5
Glutathione	3		3	3	3
N-acetyl histidine (c)		30			
Allopurinol	1				
α- Ketoglutarate		2			
Aspartate		8			
Deferoxamine/L20iron chelator		0.5/0.02			

HES, hydroxyethylstarch; HTK, histidine–tryptophan–ketoglutarate; OPS, organ preservation solutions; PEG, polyethylene glycol.

(a) PO_4^{3-} is both anion and buffer.

(b) Lactobionate is anion with calcium chelation properties.

(c) N-acetyl histidine is an osmolyte and intracellular buffer.

(*) For composition, see Figure 4B

(**) For composition, see Figure 4A

Table 3: The systemic capillary forces

	Systemic	Hypot.Perf protocol
outward flow	mmHg	mmHg
P_c	17	4.2
π_{isf}	5	5
P_{isf} (negative)	6.3	6.3
total	28.3	15.3
inward flow		
P_{isf} (positive)	-	-
π_{pl}	28	15
total	28	15
net outward pressure	0.3	0.3