## Review Article

# Extracorporeal Perfusion of Isolated Organs of Large Animals – Bridging the Gap Between *In Vitro* and *In Vivo* Studies

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**Summary**

Since the early 20th century, extracorporeal perfusion of large animal organs has been used to investigate a broad variety of research questions, thereby overcoming common drawbacks of *in vitro* studies without suffering from ethical concerns associated with live animal research. The technique is in accordance with the 3R principles and represents an excellent opportunity to investigate the physiology of organs in detail under standardized conditions. It is also suitable for the translation of basic pre-clinical research into a more relevant arena prior to or avoiding live animal research altogether. Furthermore, organ perfusion has also been an important tool in developing methods of organ preservation for transplantation surgery. Yet, due to the nature of the experiments, only short-term observations can be made and while cells are still exposed to their regional secretome, the whole organ itself is isolated from the body and correlations between organ systems cannot be taken into consideration. This review gives an overview over the history of extracorporeal perfusion of large animal organs and limbs, highlighting major achievements in the field and discussing different experimental set-ups. Advantages and limitations of the technique are presented. Prospective future research perspectives, which may include tracking of specific cell types and study of their distinct behavior towards different stimuli, are given to illustrate the relevance of this method.

Keywords: extracorporeal perfusion, large animal, research model, isolated perfused organ

## 1 Introduction

Scientific approaches adopted to investigate research questions in physiology or pathophysiology reach from cell cultures on one side to *in vivo* experiments on the other. There are numerous advantages associated with *in vitro* cell culture based research. The population of cells is very homogenous if they are sourced from the same individual and tissue. Experiments can be carried out in a strictly controlled and replicable environment, and costs are comparatively low, allowing for multiple replicates. This can rarely be achieved in *ex vivo* or *in vivo* set-ups due to higher costs, limited tissue availability and ethical concerns. Cultured cell lines allow research on species-specific cells; this is of particular advantage for modelling human disease, especially if primary cell lines closely resemble the situation in the donor. However, primary (human) cell lines may not always be available for the disease of interest. *In vivo* and *ex vivo* experiments are usually limited to animal species and extrapolation from such research to the human disease is often difficult. Disadvantages of cell culture experiments include the isolation of cells from their natural environment and the resulting lack of physiological cell-to-cell contact and mediators (Caron et al., 2012). On the other hand, *in vivo* experiments best reflect the complex physiological environment, and accordingly they are

**Abbreviations**

ATP, adenosine triphosphate; CD11b, cluster of differentiation molecule 11b; CPB, cardiopulmonary bypass; ECP LA, extracorporeal perfusion of large animal organs; HLA-DR, human leukocyte antigen-antigen D related (receptor); ILP, isolated limb perfusion; LDH, lactate dehydrogenase; $Na^+/K^+$ ATPase, sodium-potassium adenosine triphosphatase; NADH, reduced form of nicotinamide adenine dinucleotide; $pCO_2$, partial pressure of carbon dioxide; $pO_2$, partial pressure of oxygen; $SO_2$, oxygen saturation

Received November 29, 2016;
Accepted April 28, 2017;
Epub May 3, 2017;
doi:10.14573/altex.1611291

nearly universally used in the later stages of clinical research to confirm earlier results from *in vitro* studies. However, even genetically similar animals display individual differences, thus requiring research on a considerable number of animals and consequently long and costly experimental set-ups affecting animal well-being (Gruber et al., 2004).

One of the methods aiming to close the gap between the two aforementioned techniques is the use of *ex vivo* tissue explants. In explant cultures, small pieces of tissue are harvested and cultured as a whole; this retains both the microenvironment of the resident cell populations as well as 3D structure and natural matrix. However, explant cultures lack blood circulation and regional secretome exposure. In addition, the process of harvesting such tissue explants induces inflammation, diminishing the ability to accurately predict *in vivo* outcomes (McCoy, 2015). Compared to tissue explants, extracorporeal organ perfusion further approximates the *in vivo* environment, with organ specific cells maintained in their natural 3D structure and provision of a functional blood supply. By using abattoir-derived specimens, live animal research may be avoided or at least further reduced, given the possibility of detailed management of perfusion parameters allowing greater refinement of later *in vivo* experiments. However, the genetic heterogeneity of organ donors may also influence experimental data variability and, as with *ex vivo* tissue explants, inflammatory changes may occur during the harvest, transport, and storage of organs, as during extracorporeal perfusion, e.g. during cardiopulmonary bypass (CPB) surgery where blood is pumped through an extracorporeal system (Butler et al., 1993). Isolated perfused organs retain their functional blood supply but lack the complex interplay of organs and metabolic features of a live organism, e.g., hepatic metabolism, nociception or febrile responses. Isolated perfused organs therefore do not reflect the *in vivo* situation in its full complexity.

Extracorporeal perfusion of large animal organs (ECP LA) has been successfully performed for over a century. The possibility to harvest organs at an abattoir without affecting the commercial use of animals has allowed large studies to be carried out, e.g., in one study 492 organs (including hearts, lungs, livers and kidneys) were perfused and analyzed (Grosse-Siestrup et al., 2002a). Although it is possible to harvest multiple organs at the same time, requirements for staff and equipment commonly limit experiments to the simultaneous perfusion of one to two organs.

In veterinary science, isolated limb perfusion (ILP) has facilitated new insights, e.g., into the clinical picture of laminitis (Wüstenberg, 2006; Patan-Zugaj et al., 2012, 2014), a severely debilitating, painful and potentially life-threatening equine and bovine disease. Due to its significant impact on animal well-being and the complexity of potential causative agents and risk factors, continued research efforts are still needed. While few studies have used live animals, the justification to induce such a painful condition in live animals may be challenged. The sample sizes required for scientific validity and achieved in studies utilizing organ perfusion methods (e.g., n = 10 (Patan-Zugaj et al., 2012), n = 14 (Patan-Zugaj et al., 2014)) would certainly not have been justifiable *in vivo*.

Although the focus of this review is on the perfusion of organs of large animals, it should be mentioned that the same technique has been very successfully applied in small animals. Extracorporeal organ perfusion in small animal species was carried out predominantly in rats (Bounakta et al., 2017), but also in rabbits (VanGiesen et al., 1983), Guinea pigs (Kleber, 1983), dogs (Kitaguchi et al., 1979), cats (Hebb and Linzell, 1951), as well as mice (Wang and Wang, 2005). Rat organs that have been perfused include hearts (Yue et al., 2000), liver and intestines (Hager and Kenney, 1968; Windmueller et al., 1973), kidney (Nishiitsutsuji-Uwo et al., 1967), limbs (Hicks et al., 1980), and lung (Seibert et al., 1993).

The aim of this review is to describe the historical development of large animal organ perfusion for research, to compare and contrast the methods currently used in ECP LA and to indicate which research interests are best suited for which organ perfusion technique. Furthermore, the most pertinent findings obtained applying this methodology are presented.

## 2 Methodologies used for extracorporeal perfusion of large animal organs

English and German articles used for this review were identified using the search engines PubMed and Google Scholar. Keywords used were “perfusion/perfused”, “isolated”, “extracorporeal”, “preservation”, “*ex vivo*” and “animal model”. Additional materials referenced in the initially identified articles were also included where suitable. The authors focused on research conducted in large animal organs (bovine, porcine, equine, ovine, and caprine). In case the same conclusion was drawn in multiple articles, a selection of references was used.

The following nomenclature is used: the term “perfusion” describes a technique in which organs are separated from the body’s blood circulation but maintained under physiological conditions using an extracorporeal artificial circuit. Specimens used in these experiments are termed “organs”, which includes internal organs as well as limbs, muscles, skin, and udder unless otherwise specified. The fluid serving as the blood equivalent in perfusions is referred to as “perfusate” and the pressure, flow rate and temperature the perfusate is administered at are termed “perfusion pressure, perfusion flow and perfusion temperature”, respectively.

In the field of perfusion, numerous set-ups have been designed and individually tailored to fit specific requirements. Next to the hardware set-up, a multitude of different variables have to be defined as well, such as perfusion flow and pressure, perfusate composition and oxygenation. It is difficult to compare findings obtained in different species and organs on the basis of varying experimental techniques and scientific aims as isolated organ perfusion represents a complex interaction between numerous different factors that cannot be fully assessed in isolation. However, an outline of commonly applied methods in ECP LA experiments may serve to demonstrate the range of possibilities and inform decisions regarding future use of this methodology.

The authors propose that as a minimum the following information should be reported for extracorporeal perfusion experiments in order to allow other researchers to comprehend the conducted experiments and potentially replicate results: donor selection, organ collection and transport, perfusion fluid (oxygenation system, perfusion flow and pressure, temperature, type, additives), and viability measurements, such as edema formation, oxygen uptake and glucose consumption. Parameters indicative of cell death, such as levels of potassium and lactate dehydrogenase (LDH) in the used perfusate, are also recommended. Histology and organ specific parameters are of additional benefit.

### 2.1 Donor selection

To date, as research has predominantly focused on aspects of physiology or organ preservation techniques, specimens from healthy, large animals have mostly been used. Specimens of healthy animals can be sourced from abattoirs, thereby supporting 3R principles (reduce, refine, replace).

The collection of naturally diseased organs represents a promising future step, however it requires greater flexibility of the research team, as the availability of such organs is typically difficult to predict and diseased animals will not commonly be presented to local abattoirs. Such an approach may therefore prove challenging with regard to coordinating staff availability and preparation time.

The use of organs from animals that have had disease induced in the course of (other) experiments and have reached their study endpoint would be an alternative approach to obtaining diseased organs. In most terminal experiments, organs that are not central to the primary investigation are discarded. Harvesting these organs would make better use of available resources and allow modelling of advanced disease stages. However, close links between research groups are required in order to effectively utilize this resource.

Finally, induction of disease in live animals for the sole purpose of using their organs *post mortem* could also be considered. While this would still reduce the burden on animal well-being and facilitate structured and controlled experiments, it negates one of the core 3R principles: The replacement of animal use altogether.

The decision whether to use euthanized or slaughtered animals would depend on the perfusion set-up and research question. In euthanized animals, the blood volume available for perfusion is smaller, as animals are not stunned and exsanguinated, rendering autologous blood perfusion difficult to achieve. Moreover, potential drug interactions between euthanasia solutions (typically barbiturates) and organ performance during perfusions remain a concern. To the authors' knowledge, these questions have not been addressed so far and would require answering to fully promote use of diseased organs harvested after euthanasia.

### 2.2 Organ collection and transport

Organs should be harvested as soon as possible after death to avoid excessive ischemia/reperfusion injury (Blaisdell, 2002). As tissues show variable susceptibility towards ischemia, the critical time window varies depending on which organs are harvested (Steinau, 2013). Limbs represent a particular challenge, as specimens have a critical time window of only four hours owing to the skeletal muscle (Blaisdell, 2002).

Arteries used for perfusion must be cannulated swiftly, and larger arteries not used for perfusion must be occluded to reduce retrograde perfusate flow. If a closed system is used, major veins also require cannulation with the remaining veins being occluded. In open systems, veins remain open and the venous return drips into a reservoir or sampling container. Once catheters have been placed, specimens should be either connected to the perfusion circuit or, if transportation of the specimen is required, flushed with cold preservation solution. Either way, the first fluid used for perfusion should always contain an anticoagulant to prevent thrombus formation. If temporary storage or transport of the organ is necessary, the duration of warm ischemia should be kept as short as possible. Therefore, after the cold flush, specimens should also be cooled externally to further reduce metabolism (Patan et al., 2009). This is best achieved by wrapping specimens with swabs soaked in electrolyte solution or by complete immersion in a plastic bag and storage on ice (VanGiesen et al., 1983). While this is suitable for many internal organs, practical considerations might not always allow this method, e.g. when skin contamination of limb specimens is a concern.

During ischemia, a decrease in adenosine triphosphate (ATP) synthesis and oxidative phosphorylation lead to detrimental intracellular changes (disruption of homeostasis, increased membrane permeability, activation of hydrolases) and ultimately to cell death. This process is markedly slower if organs are kept between 4 and 0°C (De Groot and Rauen, 2007). Upon reperfusion of the organ, an inflammatory response is triggered by cell debris and/or altered tissue matrix as a result of anoxic cell injury; this may also be triggered by cold-induced iron ion-dependent apoptosis (e.g., endothelial cells, renal tubular cells, hepatocytes). So, despite showing the same ischemic injury (at different rates), reperfusion injury and inflammatory response are distinct after warm or cold ischemia (De Groot and Rauen, 2007). Complex preservation solutions containing protective compounds such as iron chelators (to reduce cold-induced apoptosis) and with low levels of sodium (to re-establish cell homeostasis) reduce the problems associated with reperfusion injury (De Groot and Rauen, 2007). The preservation solution should be formulated to the same specifications as the perfusate as inadequate oncotic pressure may lead to edema formation even before ECP LA has been initiated (Drapanas et al., 1966).

### 2.3 Initiation of perfusion following transport and data collection

Upon arrival in the laboratory, the organ should be connected to the perfusion system as soon as possible to limit the duration of ischemia (Fig. 1). An equilibration period with a low flow rate that is gradually increased allows the specimen to warm slowly and prevents edema formation (Wüstenberg, 2006; Patan et al., 2009).

Organ viability should either be measured continuously or at least measured in short intervals to permit early intervention and

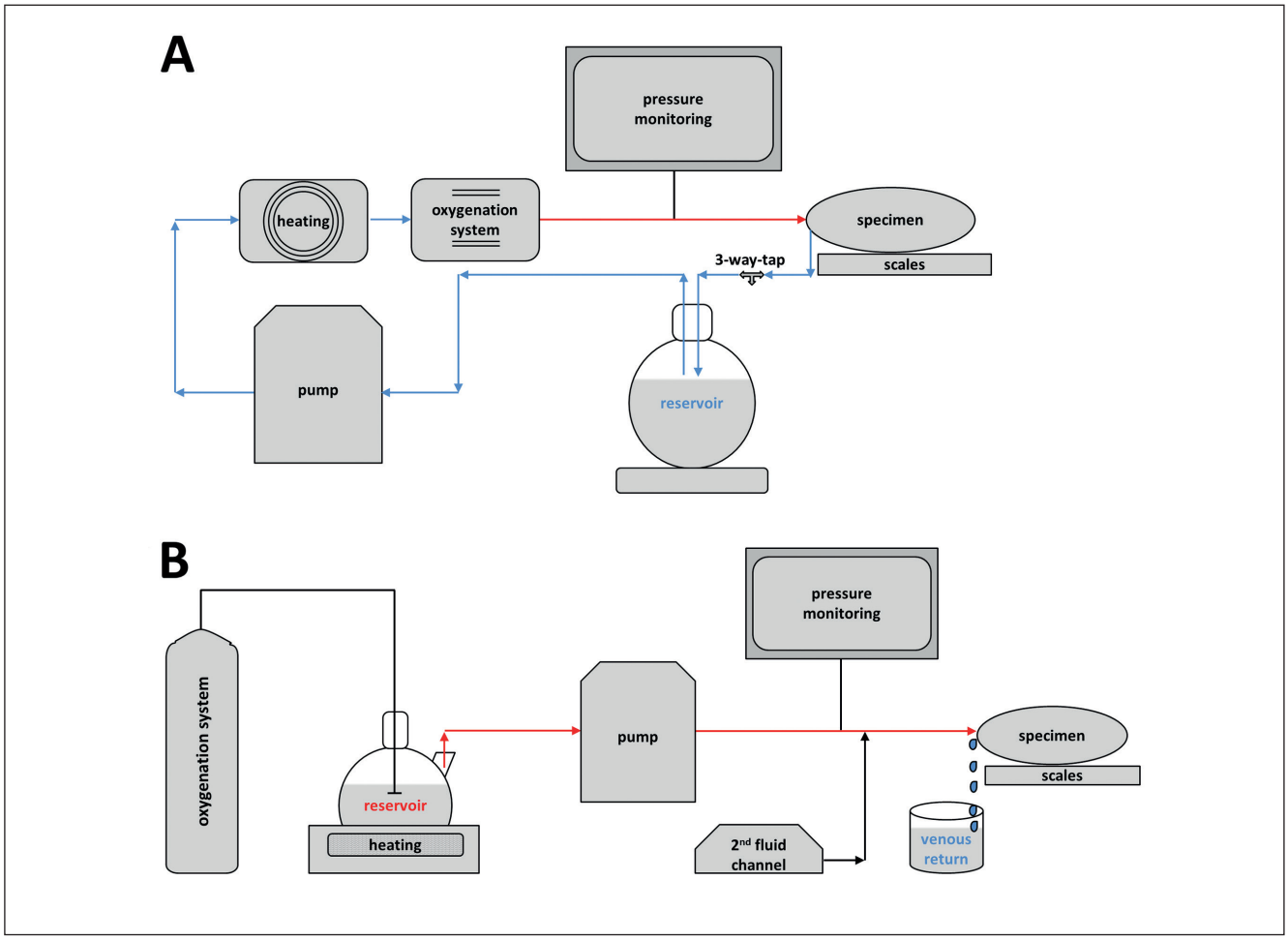


**Fig. 1: Potential set-up of a closed and an open perfusion system**

A, Closed perfusion system: The perfusate is stored in a venous reservoir and propelled (blue arrows) by a pump to a heat exchanger (e.g. a water bath), which warms the perfusate to body temperature. The perfusate flows through an oxygenation system, e.g., a hollow fibre membrane oxygenator. The oxygenated perfusate (red arrow) is pumped through the specimen's main arteries, capillary network and venous system. A device for measuring the perfusion pressure is connected to the circuit. The venous return is fed back into the system via the cannulated main veins of the specimen. The specimen rests on scales to assess edema formation during perfusion. Perfusate samples are taken via 3-way taps. B, Open perfusion system: An oxygen source is connected to the arterial reservoir and gasses the fluid, e.g., via a sintered glass filter. The reservoir is warmed to body temperature by a heating device (e.g., a water bath or hot plate). A pump propels (red arrows) the solution through the main arteries, capillary network and venous system of the specimen. The venous return (blue drops) drips freely into a beaker and can be collected at that point. The specimen rests on scales to assess weight gain. A pressure monitoring system is connected to the system to inform on perfusate pressures. A second controlled fluid channel is formed by another pump system and could introduce select additives into the system.

the correction of parameter deviations (e.g., potassium concentration, blood pressure, and pH). This is especially important for measurements in which changes over time are of interest. Tissue samples are usually collected following conclusion of the perfusion period, but careful sampling during the perfusion period may also be possible.

## 2.4 Perfusate

The choice of the most suitable perfusate depends on the specific research interest, existing resources, and availability. While autologous blood is the most physiological choice, availability, coagulation and the cellular damage created by the mechanical perfusion system limit its use, and other isotonic, isooncotic solutions that adequately sustain physiological functions may be preferred.

*Oxygenation*

In order to meet the tissues' oxygen demand, most perfusion set-ups use gas-enriched perfusates. A sufficient oxygen supply guarantees availability of ATP as an aerobic energy source, protecting and sustaining mitochondrial function. As mitochondria are involved in signaling pathways responsible for apoptosis

and necrosis, their protection leads to a reduction in cellular damage and therefore better preservation and (re)transplantation outcomes (Fuller and Lee, 2007).

Early experiments in isolated perfused udders incorporated isolated perfused lungs with the trachea connected to a gas source in the circulatory system. In this system, the perfusate flow was directed from the lung parenchyma to the isolated organ, mirroring normal physiology. The maximum perfusate flow rate achieved by this technique compared favorably with *in vivo* rates of blood flow (Hebb and Linzell, 1951). However, this complex set up has not been used since, and lungs have subsequently been replaced by other means of oxygenation (Hardwick and Linzell, 1960).

The simplest and most widely adopted gas enrichment method is to channel gas into the perfusate reservoir prior to its circulation through the system. This approach was most likely used in various experiments in which the publications solely mention the use of "gassed" (Kietzmann et al., 1993) or "oxygenated" (Bäumer and Kietzmann, 1999) perfusates without further specification of the oxygenation system. Perfusate reservoirs may be gassed using glassware with a sintered glass filter (Tindal, 1957), with a bubble oxygenator, which bubbles (pure) oxygen through the blood reservoir (Smith et al., 1985), or by using a rotating disc oxygenator (Drapanas et al., 1966; Cameron et al., 1972), whereby several parallel discs rotate within the reservoir containing unoxygenated blood and the gas exchange occurs at the disc-blood interface. More recently, the use of membrane or hollow fiber oxygenators (Butler et al., 2002; Müller et al., 2013) typically deployed during CPB surgery or life support (extracorporeal membrane oxygenation) has been reported in perfusion experiments. These systems generally follow the same concept as rotating disc oxygenators, as oxygen exchange occurs at a porous membrane. A simple but effective adaptation of this principle is the use of gas-permeable tubing exposed to room air in a custom made oxygenation chamber, which has been shown to be effective for oxygenation of media containing erythrocytes (Hamilton et al., 1974; Patan et al., 2009).

The most frequently used gas is carbogen (95% $O_2$ and 5% $CO_2$) as described in Kietzmann's model (Kietzmann et al., 1993). Carbogen may be chosen when the buffer capacity of commonly used cell-free perfusion solutions depends on the presence of $CO_2$ (Mancina et al., 2015) or when using solutions without oxygen carriers, as these require a higher $O_2$ content than in room air to meet the tissues' oxygen demand. Pure oxygen is rarely used in perfusion experiments (Drapanas et al., 1966) as unphysiologically high oxygen tension may lead to the production of free radicals (Fuller and Lee, 2007) and a lack of buffer capacity of $CO_2$ may cause a pH drop. Carbogen of varying relative $CO_2$ concentrations has been used (2.5% (Grosse-Siestrup et al., 2002b) to 7% (Hardwick and Linzell, 1960)).

For perfusates containing erythrocytes, such as blood/plasma (Patan et al., 2009) or blood/dialysate mixtures (Wagner et al., 2003), room air is adequate to achieve satisfactory saturation of the perfusate while a low flow rate may support sufficient time for gas exchange in the oxygenation system (Wagner et al., 2003).

Recently, specific perfusion solutions not requiring an oxygenation system have been tested. Polyak et al. (2008) perfused equine large colon with either an adapted cell free preservation solution or oxygenated whole blood. Organs perfused with the novel solution relied on ambient air as their sole source of oxygenation. The novel perfusate prevented edema (e.g., via modified hydroxyethyl starch, and mannitol), provided cells with adequate energy (e.g., via ATP intermediates and dextrose), reduced the inflammatory response (e.g., via prostaglandin $E_1$ and L-glutamine), and improved vascular and biochemical variables (Polyak et al., 2008). Not needing an oxygenation system may simplify the perfusion set-up, but developing and testing a highly complex perfusate is labor- and cost intensive and might not be practical in every instance.

*Pressure and flow rate*

Hemodynamic parameters, namely blood pressure (P, $\Delta P = P_{arterial} - P_{venous}$), blood flow (F), and vascular resistance (R) are interdependent as described by Ohm's law:

$$F = \frac{\Delta P}{R}$$

While this equation applies to laminar flow conditions and thereby represents a simplified view of normal blood flow, it forms the basis for the artificial circuit set-up.

Perfusion can either be controlled by flow rate or by pressure, as the flow rate is adjusted to reach a certain pressure, or vice versa. Flow controlled set-ups have been used most often (Chapman et al., 1961; Villeneuve et al., 1996; Grosse-Siestrup et al., 2002b; Zeitlin and Eshraghi, 2002; Dragu et al., 2011), but newer evidence suggests that pressure controlled perfusion improves organ preservation (Mancina et al., 2015). Two strategies may be applied in flow controlled experiments: a low flow or a high flow approach. In low flow circuits, the average flow rate and consequently the perfusion pressure, is below physiological values; however the flow still reaches and supplies the capillary bed, as demonstrated using dyes (Kietzmann et al., 1993), specimens remain viable for up to twelve hours (Cypel et al., 2008; Müller et al., 2013), and microvasculature impairment and hydrostatic edema formation (Cypel et al., 2008; Constantinescu et al., 2011; Müller et al., 2013) is reduced in comparison to high flow approaches. Systems which employ a high flow approach aim for physiological flow rates and perfusion pressures to mimic the *in vivo* situation more closely. As a higher flow rate leads to lower organ resistance, this indicates the integrity of blood vessels (Barthel et al., 1989; Bristol et al., 1991; Wagner et al., 2003). Lactate levels in venous perfusate samples obtained during high flow experiments are lower than in low flow experiments and this could also imply a better oxygenation of tissues (Wagner et al., 2003).

Although early experiments suggested that there is no difference in pulsatile *versus* constant flow (Pegg and Green, 1976), other groups have shown pulsatile flow to be superior compared to a steady flow of perfusate (Vang and Drapanas, 1966; Finn et al., 1993; Sezai et al., 1999). In CPB experiments, pulsatile perfusion using hydraulically driven dual-chamber physiological pulsatile pumps enabled better

cerebral blood flow with lower resistance than with traditional roller pumps. This might be due to higher hemodynamic energy being generated in the pulsatile perfusion (Ündar et al., 2002). Overall, a true pulsatile perfusion reflects the physiological situation better than non-pulsatile set-ups. Nevertheless, the use of roller pumps might still be valuable for research applications as they produce good results in ECP LA and allow more cost-effective set-ups.

*Temperature*

Perfusates may be hypothermic or normothermic. Hypothermic perfusions are adopted for isolated organ and limb perfusions with the aim to promote organ preservation and facilitate reimplantation (Smith et al., 1985; Domingo-Pech et al., 1991; Guarrera et al., 2004; Constantinescu et al., 2011; Müller et al., 2013). Hypothermia slows cellular metabolism and oxygen consumption, allowing lower flow rates and perfusates without specific oxygen carriers to meet metabolic requirements (Fuller and Lee, 2007). Using lower flow rates may better preserve microcirculation (Cypel et al., 2008) and prevent edema formation (Constantinescu et al., 2011). Other authors however describe weight gain and increased organ resistance due to uncharacteristic sheer forces and viscosity of cold solutions as drawbacks of hypothermic pressure-driven flow rate perfusions (Fuller and Lee, 2007). This weight gain however might be the result of several components working together rather than being solely attributed to the hypothermic perfusion itself. Low temperatures may also lead to reduced antioxidant defenses and subsequently to production of free radicals and toxic concentrations of superoxides within tissues (Fuller and Lee, 2007). In contrast, it is known from extracorporeal perfusion during CPB that hypothermia eases inflammation in the brain (Schmitt et al., 2007). During hypothermia, neutrophil and monocyte migration is markedly reduced (Biggar et al., 1984), which undoubtedly reduces inflammatory responses but might also lead to a higher infection rate.

Reduced metabolism and altered inflammatory responses may render a hypothermic approach less suitable for most research applications. Indeed normothermia is pursued in almost all scientific implementations of the methodology (Kietzmann et al., 1993; Patan-Zugaj et al., 2012). For studies with a physiological, pharmacokinetic or pharmacologic focus (Roets et al., 1979b; Bäumer et al., 2002; Friebe et al., 2013b), the use of hypothermia would seem contra-indicated.

*Blood based solutions*

In its complex composition, blood provides perfectly balanced components as well as the most suitable oncotic pressure. This is reflected in relatively low weight gain from edema formation in experiments using whole blood as perfusate (Patan et al., 2009; Constantinescu et al., 2011; Mancina et al., 2015). However, one report shows a higher weight gain in limbs perfused over five hours with diluted autologous blood (blood: Tyrode solution 4:1) compared to limbs perfused over eight hours with adapted Tyrode solution containing sodium carboxymethyl cellulose (0.15 g/l) (Friebe et al., 2013b). Whole blood also contains erythrocytes which represent an optimal oxygen carrier and therefore enable sufficient oxygen transport to perfused tissues (Mancina et al., 2015).

Instead of autologous blood perfusions, several experiments have utilized homologous blood or blood components with no apparent negative effects (Hebb and Linzell, 1951; Cameron et al., 1972; Butler et al., 2002). While this may simplify the experimental set-up and potentially reduce inter-animal variability, it is plausible that incompatibility reactions can occur, thereby affecting outcome variables. Until further evidence for this approach can be provided, the use of autologous blood may therefore be preferable.

Slightly greater data variability may be encountered compared to perfusions with fully artificial solutions, due to inter-individual differences in the animals' blood composition (Tab. 1).

Unmodified blood is not suitable for perfusion purposes, as at least the addition of anticoagulants is necessary for its use. It may also be recommended to dilute whole blood as this decreases its viscosity and thus improves blood flow. This applies particularly to the capillary bed, where blood flow is comparatively slow and shear rates are low. As blood is a shear thinning non-Newtonian fluid, its viscosity depends on shear rates. Where shear rates are low (as in the capillary bed), blood viscosity is highest. So, despite the reduced concentration of oxygen carriers due to its dilution, the improved flow rate leads to better tissue supply and consequently to superior organ function (Dittrich et al., 2000). For dilution purposes, plasma (Patan et al., 2009) or artificial solutions such as Tyrode solution (Friebe et al., 2013b), modified Krebs-Henseleit buffer (Mancina et al., 2015) or dialysate solution (acetate hemodialysis concentrate HD 22, Fresenius Medical Care, Bad Homburg, Germany) (Wagner et al., 2003) have been used successfully.

Disadvantages associated with the use of whole blood mainly pertain to the logistical challenges associated with the harvest and processing to render it a suitable perfusate; this is certainly more demanding as well as more time-sensitive compared to the preparation of cell free solutions. Furthermore, stress that animals may experience *ante mortem* could change the blood composition, e.g. adrenalin release (Persson, 1967). If autologous blood is to be used, it is typically harvested at the time of or slightly prior to death, requiring both blood and tissue specimens to be processed at the same time. For homologous perfusions, blood donors may be utilized independent of the euthanasia of organ donors. The limited volume of available blood might require use of a recirculating system to ensure perfusion at adequate flow rates over the desired length of time. However, closed or recirculating systems are also associated with damage to blood cells (Watanabe et al., 2007; Lee et al., 2007) and metabolic waste products such as lactate will accumulate over time (Bristol et al., 1991). Furthermore, the use of blood collected from animals under anesthesia or following euthanasia contains drug residues that may affect blood physiology (Barlow and Knott, 1964; Gentry and Black, 1976; Honkanen et al., 1995). Therefore, use of

**Tab. 1: Applications for blood based and artificial perfusion fluids**
The choice of a specific perfusion fluid depends on the perfused organ and the research question. This table gives a short overview of select applications of either blood based or artificial perfusion solutions. For specific recipes, compounds and additives to optimize the fluid's performance, refer to the respective publications.

| Perfusion fluid | Organ | Species | Research area | Research group / Select publications |
|---|---|---|---|---|
| **Blood based** | Heart | Porcine | Pathophysiology | Janse et al., 1980 |
| | Kidney | Porcine | Organ preservation | Grosse-Siestrup et al., 2002a |
| | Limb | Equine | Pathophysiology | Patan-Zugaj et al., 2012, 2014; Patan et al., 2009; Gauff et al., 2013, 2014 |
| | | Porcine | Pharmacology | Wagner et al., 2003 |
| | Liver | Bovine | Organ preservation | Chapman et al., 1961 |
| | | Porcine | Perfusion | Adham et al., 1997 |
| | | | | Butler et al., 2002 |
| | | | | Grosse-Siestrup et al., 2002a,b |
| | | | Physiology | Cameron et al., 1972 |
| | | | | Drapanas et al., 1966 |
| | Lung | Porcine | Organ preservation | Erasmus et al., 2006 |
| | Udder | Bovine | Physiology | Cowie et al., 1951; Verbeke et al., 1959; Lauryssens et al., 1959, 1960, 1961; Wood et al., 1965 |
| | | Caprine | Physiology | Hebb and Linzell, 1951; Hardwick and Linzell, 1960; Hardwick et al., 1961, 1963; Hardwick, 1965, 1966; Linzell et al., 1967; Verbeke et al., 1957, 1968, 1972; Roets et al., 1974, 1979a,b |
| **Artificial** | Heart | Porcine | Perfusion | Chinchoy et al., 2000 |
| | | | Physiology | Araki et al., 2005 |
| | | | | Coronel et al., 1988 |
| | Intestine | Equine | Organ preservation | Polyak et al., 2008 |
| | | Porcine | Physiology | Hansen et al., 2000, 2004 |
| | | | | Rehfeld et al., 1982 |
| | Kidney | Porcine | Pathophysiology | Köhrmann et al., 1994 |
| | | | Organ Preservation | Mancina et al., 2015 |
| | Limb | Equine | Pharmacology | Friebe et al., 2013a,b |
| | | Porcine | Organ preservation | Constantinescu et al., 2011; Müller et al., 2013 |
| | | | Pharmacology | Wagner et al., 2003 |
| | Lung | Porcine | Organ preservation | Cypel et al., 2008 |
| | | | Physiology | Hellewell and Pearson, 1983 |
| | Udder | Bovine | Pharmacology | Kietzmann et al., 1993, 1995, 2008, 2010; Forster et al., 1999; Bäumer and Kietzmann, 1999, 2001; Ehinger and Kietzmann, 2000a,b; Ehinger et al., 2006; Schumacher et al., 2011 |

**Tab. 2: Basic composition of commonly used solutions**
Recipes vary between different research groups and suppliers. Mixtures are often adapted to closely represent species-specific plasma concentrations of components.

| Component in mM | Tyrode | Krebs- Henseleit | Krebs-Ringer (bicarbonated) |
|---|---|---|---|
| **NaCl** | 137 | 118 | 115 |
| **KCl** | 2.7 | 4.7 | 5.9 |
| **$CaCl_2$** | 1.8 | 1.3 | – |
| **$MgCl_2$** | 1.05 | – | 1.2 |
| **$NaHCO_3$** | 12 | 25 | 25 |
| **$MgCl_2$** | 1.8 | – | – |
| **$NaH_2PO_4$** | 0.42 | – | 1.2 |
| **$KH_2PO_4$** | – | 1.2 | – |
| **$MgSO_4$** | – | 1.2 | – |
| **$Na_2SO_4$** | – | – | 1.2 |
| **Glucose** | 5.5 | 11 | 10 |

blood obtained via exsanguination as part of slaughter would seem the most advantageous option. A drawback, however, is the potential lack of sterility that occurs during the harvesting process, as bacterial contamination is especially problematic in experiments of longer duration.

*Cell free solutions*
Cell free perfusion solutions are generally based on Ringer's isotonic solution containing sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), and sodium bicarbonate ($NaHCO_3$) as elementary ingredients. The solution is usually modified and enriched with various additives. The variants most commonly used for research purposes are Tyrode solution (Kietzmann et al., 1993), Krebs-Henseleit solution (Berhane et al., 2006), and bicarbonated Krebs-Ringer solution (Hansen et al., 2004) (Tab. 2).

Several specifically designed perfusion solutions have been reported in support of therapeutic organ transplantations; the most commonly used are Eurocollins solution and the University of Wisconsin solution. The former distinguishes itself by its relatively high glucose content (214 mmol/l) and is best suited for lung preservation (Hicks et al., 2006), while the latter is characterized by three impermeants (lactobionate, raffinose, and hydroxyethyl starch) and is well-matched for kidney, liver and pancreas preservation (Hicks et al., 2006). Both solutions are unsuitable for the preservation of muscle and extremities (Tsuchida et al., 2001, 2003).

As mentioned above, Polyak et al. (2008) demonstrated that the use of a novel unoxygenated artificial solution they had developed could maintain viability of equine intestine in perfusion experiments for twelve hours and was superior to the use of whole blood (Polyak et al., 2008).

One of the advantages associated with the use of cell free solutions is their relatively easy handling in a perfusion set-up compared with blood. The solution is readily available, even in large volumes that might be needed if an open system is the set-up of choice and variability in perfusate composition is not a concern. The use of cell free solutions facilitates standardization of the experimental environment, minimizes unwanted interactions and allows a refined focus on the behavior of specific cell types. A disadvantage is represented by increased edema formation due to inadequate oncotic pressure of artificial perfusion solutions (Domingo-Pech et al., 1991; Zeitlin and Eshraghi, 2002); however, weight gain of organs has also been reported in experiments using diluted whole blood as perfusate (Friebe et al., 2013a).

*Additives*
A broad variety of perfusate additives has been reported, and the most commonly used additives in the context of experiment types, specimens and species are presented here (Tab. 3). Additives that are infrequently used and that are highly specific to organs or research questions are not covered.

The use of whole blood perfusates requires the addition of an appropriate anticoagulant to prevent thrombus formation and consequent disturbances in blood flow; furthermore, anticoagulants may lower the inflammatory response associated with ischemia/reperfusion injury, as clotting plays an important role in this process (Blaisdell, 2002). Heparin, an inhibitor of antithrombin III (Hirsh et al., 1995), is the most widely used anticoagulant (Hardwick et al., 1961; Patan et al., 2009; Constantinescu et al., 2011). In addition to volume expansion, dextran solutions also contribute thrombolytic and antithrombotic properties (Fischer et al., 1985; De Raucourt et al., 1998), rendering

them popular additives to perfusates (Tindal, 1957; Drapanas et al., 1966; Cameron et al., 1972; Rehfeld et al., 1982; Domingo-Pech et al., 1991; Kietzmann et al., 1993; Hansen et al., 2000).

Vasoconstriction, a core response in reperfusion injury, may be counteracted by the addition of vasodilators such as nitroglycerin (Domingo-Pech et al., 1991) or prostacyclin (Erasmus et al., 2006), which improve flow rates. Especially in udder perfusion studies, when aspects of milk production are at the center of interest and relatively high flow rates are required, blockage of 5-hydroxytryptamine induced vasoconstriction has been shown to be advantageous (Roets et al., 1974, 1979a,b). Improving flow rates, especially when using whole blood perfusates, alters the perfusate's rheological properties. This prompted research groups to hemodilute perfusates to modify blood viscosity and reduce vascular resistance, a technique widely applied in CPB procedures (Dittrich et al., 2000). As indicated above, this approach improves tissue oxygenation despite the reduced concentration of erythrocytes (Dittrich et al., 2000). Reported diluents have been cell free solutions (Domingo-Pech et al., 1991; Wagner et al., 2003; Erasmus et al., 2006; Friebe et al., 2013b) or autologous plasma (Patan et al., 2009; Patan-Zugaj et al., 2012, 2014), which comes with the added benefit of increasing oncotic pressure. As cell free solutions are associated with low oncotic pressure, resulting in edema formation and weight gain, adding plasma expanders such as plasma proteins (Verbeke et al., 1968; Roets et al., 1974; Patan et al., 2009) and purified albumin (Rehfeld et al., 1982; Barthel et al., 1989; Riviere et al., 1989; Brunicardi et al., 2001) may be necessary. Plasma expanders/replacers used for clinical and research purposes and showing a similar effect are dextran (Cameron et al., 1972; Kietzmann et al., 1993), cellulose (Friebe et al., 2013a), hydroxyethyl starch (Müller et al., 2013) and mannitol (Domingo-Pech et al., 1991; Labens et al., 2013). While mannitol is considered an inert substance, recent research has suggested possible interdependencies with monocyte and neutrophil function (upregulation of HLA-DR in monocytes, upregulation of CD11b in neutrophils and monocytes, and inhibition of neutrophil apoptosis (Turina et al., 2008)). Hydroxyethyl starch (Handrigan et al., 2005; Matharu et al., 2008) and cellulose (Hänsch et al., 1996; Moore et al., 2001; Bae et al., 2004; Ewoldt et al., 2004; Hernández et al., 2004, 2009) have also both been shown to alter inflammatory responses due to their different effects on neutrophils.

While glucose is also able to support the maintenance of oncotic pressure (Hicks et al., 2006), it supports the specimen's metabolism and survival. Glycolysis represents the main energy source for living tissue, ensures ATP production under anaerobic and aerobic conditions (Silbernagl and Despopoulos, 2012) and is consumed by specimens in perfusion experiments. Glucose is therefore an often used additive in blood based solutions (Butler et al., 2002; Patan et al., 2009) and part of the basic recipe of artificial fluids. Closed systems require a regular compensation for glucose loss.

Oxygen carriers are often added to artificial solutions. Isolated erythrocytes, as the body's natural oxygen carriers, are very well suited for this purpose although their use in closed systems entails the risk of mechanical cell damage over time (Watanabe et al., 2007; Lee et al., 2007). In early experiments

**Tab. 3: Most commonly used additives in perfusion experiments**

| Additive | Class | Function |
|---|---|---|
| Heparin | Anticoagulant | Prevention of thrombi |
| Dextran | Anticoagulant | Prevention and lysis of thrombi |
| | Impermeant | Prevention of edema |
| Penicillin and streptomycin | Antimicrobial agents | Prevention of bacterial overgrowth |
| Sodium bicarbonate | Buffer | Maintenance of pH levels |
| Methylprednisolone | Glucocorticoid | Prevention of general inflammation (reduction of vascular leakage) |
| Plasma proteins/ Serum albumin | Impermeant | Prevention of edema |
| Glucose | Impermeant | Prevention of edema |
| | Metabolic substrate | Maintenance of metabolism |
| Aminoacids | Metabolic substrate | Maintenance of metabolism |
| Erythrocytes | Oxygen carrier | Enhancement of oxygen delivery and maintenance of aerobic metabolism |
| Cell free solutions | Rheological active agent | Hemodilution |
| Nitroglycerin or prostacyclin | Vasodilator | Improvement of vascular flow |

fluorocarbons were tested with equivocal results (Usui et al., 1985; Smith et al., 1985).

Metabolic activity is also influenced by the perfusate's pH and *vice versa*. Acidosis, which might occur due to hypoxemia or hypoperfusion, leads to an increased level of lactic acid (Silbernagl and Despopoulos, 2012). This is initially buffered by alkali substances such as $NaHCO_3$, and because of this such substances are also ingredients of perfusates (Cameron et al., 1972; Riviere et al., 1989; Domingo-Pech et al., 1991; Mancina et al., 2015). Some authors report on gas flow rate to adjust partial pressure of carbon dioxide ($pCO_2$) levels and pH (Cypel et al., 2008). In most older perfusion set-ups, however, precise monitoring and adaptation of gas flow rates and $CO_2$ content were technically challenging.

Antibiotics have been used in select applications to fight bacterial overgrowth in perfusates and specimens. Most commonly a standard combination of β-lactam antibiotics (usually penicillin (Hardwick et al., 1961; Riviere et al., 1989; Bristol et al., 1991)) and aminoglycosides (usually streptomycin (Hardwick et al., 1961) or gentamicin (Riviere et al., 1989; Bristol et al., 1991)) were chosen. The drugs were applied via the perfusate either constantly (Riviere et al., 1989; Domingo-Pech et al., 1991) or intermittently (Butler et al., 2002). However, many studies have been successful without the use of antibiotics. One study with a sterile harvesting process and sterile system reported no bacterial contamination for up to 24 hours (Chapman et al., 1961). However, most perfusion experiments are conducted in an unsterile environment.

Glucocorticoids are commonly used during CPB (Varan et al., 2002) to suppress complement activation and cytokine production (Butler et al., 1993; Mold and Morris, 2001) following ischemia, which eventually leads to vascular leakage and hence to edema formation. The glucocorticoid methylprednisolone is commonly used in perfusion experiments with an interest in organ preservation (Domingo-Pech et al., 1991; Constantinescu et al., 2011) for this purpose.

### 2.5 Viability measurements

Several parameters can be measured to monitor a specimen's viability during perfusion. The following provides an overview over viability data that can be ascertained from all specimens. In addition, organ specific measurements may be carried out to obtain further detailed information about viability and tissue function, e.g., milk synthesis (Hardwick and Linzell, 1960), bile production (Drapanas et al., 1966), responsiveness to electro-stimulation (Constantinescu et al., 2011), or endocrine response (Jensen et al., 1978). In general, metabolic values are tissue specific and dependent on the mass of the perfused organ. A simple definition of the overall cut-off value for viability can therefore not be given and comparisons between studies should be made cautiously.

*Edema*

Prolonged ischemia causes alterations in the capillary bed, which ultimately leads to increased vascular permeability and therefore edema formation (Blaisdell, 2002). Weight gain of perfused specimens thus reflects the degree of ischemia/reperfusion injury (Petrasek et al., 1994; Adham et al., 1997) and the integrity of the microvasculature (Müller et al., 2013). Excessive edema formation results in rapidly progressive deterioration of organ function, which renders it unsuitable for transplantation and research alike (Verbeke et al., 1972). Next to reperfusion injury, edema formation is also promoted by inadequate oncotic pressure, perfusion pressure, or flow rate.

The easiest way to monitor edema formation is by weighing specimens before and after perfusion. While histologic assessments will also inform on edema formation, other parameters such as the measurement of skin fold thickness or the wet-to-dry weight ratio of harvested tissue samples have been used.

Hardwick and Linzell (1960) defined edema as a greater than 20% weight gain, which occurred in approximately 50% of caprine mammary gland perfusions. They pointed out that the weight gain often occurred rapidly as a terminal event at the end of five to 27 hour long experiments (Hardwick and Linzell, 1960). Kietzmann et al. (1993) however expressed concern that due to milk production in the isolated perfused udder, increased weight might not be a reliable parameter for edema formation (but might reflect milk production) and suggested that other parameters (skin fold thickness, histology) be added. They found no increase in skin fold thickness or change in histologic appearance concurrent with an average weight gain of 14% over a six hour perfusion period in that model (Kietzmann et al., 1993). Other studies have applied more stringent limits for inacceptable weight gain, leading to the exclusion of specimens with greater than ten percent increase during perfusion (Zeitlin and Eshraghi, 2002).

Perfusion of calf livers with a human blood based perfusate for up to nine hours resulted in mild to moderate interstitial edema formation (histologic assessment), which was associated with a total weight gain of 19 to 21%. Interestingly, ten percent of the observed weight gain already occurred during the initial wash-out period of autologous blood with Ringer's lactated solution (Drapanas et al., 1966). This may indicate that vascular damage or inadequate oncotic pressure was already present at the start of the perfusion experiment.

*Oxygen*

The rate of oxygen consumption by tissue can be determined applying the Fick equation:

$$VO_2 = Q\ x\ (C_aO_2 - C_vO_2)$$

The cardiac output in ml/min is represented by Q and the difference between the arterial ($C_aO_2$) and the venous ($C_vO_2$) oxygen content describes the arteriovenous oxygen difference. The arterial oxygen content represents the volume of oxygen carried per 100 ml blood and is calculated by:

$$O_2\ carried\ by\ Hb + O_2\ in\ solution$$

$$\left(1.34\ x\ Hb\ x\ \frac{S_aO_2}{100}\right) + (0.003\ x\ P_aO_2)$$

Huefner's constant (1.34) represents the experimentally measured maximum oxygen carrying capacity of hemoglobin. It is multiplied by the hemoglobin concentration (Hb) per 100 ml blood and the percentage of oxygen saturated hemoglobin ($S_aO_2$). The amount of dissolved oxygen is calculated by the product of the partial pressure of oxygen ($P_aO_2$ in mmHg) and the constant 0.003, representing the amount of oxygen dissolved in plasma. The venous oxygen content can be calculated likewise. In perfusion experiments, the organ's weight is often taken into consideration (Chapman et al., 1961; Jensen et al., 1978), e.g., Patan et al. (2009) report an oxygen uptake of $6.4 \times 10^{-6} \pm 8.9 \times 10^{-5}$ l/g/min. Measuring oxygen saturation ($SO_2$), partial pressure of oxygen ($pO_2$) and $pCO_2$ in arterial and venous perfusate samples also sheds light on the oxygen use of the perfused organ (Zeitlin and Eshraghi, 2002; Wagner et al., 2003; Polyak et al., 2008; Constantinescu et al., 2011).

*Glucose*

In living cells, glucose is converted to pyruvate during glycolysis. The released energy is used to produce the energy sources ATP and nicotinamide adenine dinucleotide (NADH). In ECP LA, consumption of glucose from the perfusate would imply functional glycolytic metabolism, producing energy to keep the specimen alive, but could also be contributed to by bacterial glucose consumption in a contaminated set-up. It has been suggested that glucose utilization ≥ 200 mg/h is indicative of a viable distal equine digit (Friebe et al., 2013a). It is however difficult to extrapolate this to other experiments as glucose metabolism is tissue-specific and also depends on the mass of the perfused organ. In closed systems, the consumption of glucose over time can be assessed by comparison of glucose levels in the arterial reservoir at different time points. To replenish glucose it is either added directly to the perfusate (Riviere et al., 1989; Wagner et al., 2003) or the arterial reservoir of the perfusate is replaced at regular intervals, thereby re-establishing prior glucose levels (Patan et al., 2009). In open systems, glucose consumption may be assessed comparing arterial reservoir contents with glucose content of the venous return from the specimen.

*Lactate*

Lactate production is increased during anaerobic glycolysis and is therefore a suitable parameter to inform on the adequacy of the oxygen delivery to tissues. In perfusion experiments an elevated lactate concentration in the outflowing perfusate or venous return may be observed immediately after the limb is connected to the circuit. This is likely due to accumulation of lactate during anaerobic transport conditions (Kietzmann et al., 1993). Increases in lactate concentration have also been observed after arterial reservoir exchanges resulted in a period of relatively low $O_2$ saturation of the perfusate due to the new reservoir solution not yet being fully oxygenated (Patan et al., 2009). Friebe et al. (1993) postulate in their minimum requirements for viability of the equine distal limb a lactate production of ≤ 400 mg/h, a criterion met by their and other groups' experiments (Patan et al., 2009). Again, these values are tissue and mass dependent and cannot be seen as absolute references. Both groups report physiological lactate levels, even if an increase of lactate levels within the physiological range was noted over the course of the experiments (Friebe et al., 2013a,b; Patan et al., 2009; Patan-Zugaj et al., 2012, 2014). In contrast, lactate levels were (constantly) elevated in the venous perfusate in porcine isolated limb perfusions (up to 105 mg/dl (Wagner et al., 2003), mean maximum value 19.59 mmol/l (Constantinescu et al., 2011)) likely due to the higher metabolic demands of a greater amount of included soft tissues.

To summarize, due to the lack of hepatic clearance a rise in lactate levels can be observed in the majority of perfusion experiments over time, even if measurements do not exceed the physiological range by the end of the experiment (Smith et al., 1985; Riviere et al., 1989; Adham et al., 1997; Polyak et al., 2008). A small decrease of lactate levels may be observed after several hours of perfusion, which may be explained by modest lactate utilization by myocytes (Constantinescu et al., 2011).

*Potassium*

Potassium levels in the perfusate may be used as an indirect measure for cell integrity and cell death (Ward and Buttery, 1979; Bortner et al., 1997). Potassium concentrations are higher in the intracellular space due to energy dependent active transport systems ($Na^+/K^+$ ATPase) maintaining osmotic balance. In the dying cell, potassium follows its electrochemical gradient to the extracellular space (Bortner et al., 1997; Trimarchi et al., 2000), thereby leading to a measurable increase in potassium in the perfusate (Polyak et al., 2008; Patan et al., 2009; Constantinescu et al., 2011). On the other hand, intracellular potassium transport indicates availability of adequate energy supplies sustained by the perfusion circuit (Patan et al., 2009). A potassium efflux of ≤ 7% has been proposed to indicate adequate cell viability during ECP LA (Ward and Buttery, 1979).

In order to control potassium levels in perfusion experiments an insulin/glucose solution may be added to the perfusate (Constantinescu et al., 2011). Insulin increases the activity of the $Na^+/K^+$ ATPase pump and excess potassium is removed from the extracellular space. However, other effects of insulin should be noted, e.g., change in endothelin-1 expression and associated laminitic changes in the perfused equine digit (Gauff et al., 2013, 2014). In the event of acidosis during perfusion experiments, potassium may also move from the intracellular space to the extracellular fluid in an attempt to buffer dropping pH levels (Oster et al., 1978). In this case an increase of potassium levels in the perfusate is initially not elicited by cell death and has to be assessed in the context of the metabolic imbalances.

*Lactate dehydrogenase*

Lactate dehydrogenase (LDH) is an enzyme responsible for the interconversion of lactate and pyruvate and thus involved

in anaerobic glucose metabolism and glucose biosynthesis (Berg et al., 2013). It can be found in almost every tissue with a tissue specific distribution pattern of its isoenzymes (Markert and Ursprung, 1962) with the isoforms LD1 and LD2 being dominant in erythrocytes (Kato et al., 2006). Therefore, elevated LDH activity may be associated with hemolysis and cell injury/death in general (Legrand et al., 1992). In perfusion experiments it has consequently been used as an indicator of cell death following hypoxemia or hemolysis (Ward and Buttery, 1979; de Lange et al., 1992; Moen et al., 1994). In whole blood perfusion experiments of the equine digit in a closed system, Patan et al. (2009) reported LDH levels in the range of 12.0 ±14.7 U/h and the authors observed a slow increase in LDH levels over a perfusion period of up to ten hours (Patan et al., 2009) indicating a good viability of the specimen. In open systems using Tyrode solution based perfusates, LDH levels tended to be lower (Kietzmann et al., 1993; Friebe et al., 2013a,b), presumably due to the lack of LDH containing erythrocytes, and have been reported to indicate adequate tissue viability of an equine distal limb during perfusion if below 10 U/h (Friebe et al., 2013a).

## 3 History and important findings

### 3.1 Transplantation, replantation and preservation

*Organs*

In the context of transplantation surgery, prolonged preservation using pulsatile perfusion has been shown to be superior to simple cold storage when assessing graft and patient survival, rate of delayed graft function and the need for post-transplant dialysis (kidney transplant) (Peter et al., 2002; Shah et al., 2008). Standardization of perfusion set-ups became possible with commercially available perfusion systems (e.g., Waters RM 3, MOX-100 (Guarrera et al., 2004)). This and the optimization of perfusion solutions (Belzer MPS/Vasosol MPS) with vasodilators (nitroglycerin, and prostaglandin $E_1$), antioxidants (polyethylene glycol-superoxide dismutase, and N-acetylcysteine), Krebs cycle intermediates (α-ketoglutarate) and metabolic substrates (L-arginine) (Guarrera et al., 2004) improved results of organ preservation dramatically. To further optimize this methodology recent studies have evaluated the difference between pressure- and flow-controlled perfusions and concluded that pressure controlled perfusions are superior with reference to renal hemodynamics and acid-base homeostasis (Mancina et al., 2015).

*Limbs*

The preservation of limbs represents a specific challenge owing to the broad range of involved tissues and their variable tolerance to ischemia (Blaisdell, 2002; Constantinescu et al., 2011).

In early ILP experiments performing canine hind limb autotransplants, the best outcome was observed implementing continuous hypothermic perfusion with solutions containing fluorocarbon as an oxygen carrier (Usui et al., 1985). Despite these initial positive results, fluorocarbon containing solutions had only moderate success during clinical trials of human digital replantation following perfusion (Smith et al., 1985). Fluorocarbon is also known to reduce neutrophil infiltration, which might be an interesting characteristic in organ preservation but could affect outcomes in research applications of the perfusion model (Forman et al., 1992).

Another study was able to demonstrate that ILP over a period of 24 hours maintained viability of amputated canine hind limbs so that after six hours post replantation limbs appeared healthy and peripheral vessels were well perfused. However, significant edema formation (20 to 50% weight gain) was observed in perfused legs requiring subsequent cooling of the perfusate and use of peripheral vasodilators and steroids (Domingo-Pech et al., 1991) to counteract inflammatory processes (Biggar et al., 1984).

In order to optimize the experimental set-up, Tsuchida et al. (2001) assessed different perfusion pressures and solutions. Comparing replantation of amputated rat hind limbs after perfusion with the superior set-up (University of Wisconsin solution, high perfusate pressure) with replantation of non-perfused limbs, outcomes with perfusion were only slightly superior. An observation that might be explained by the potential deterioration of vascular endothelial function and consequently blood flow with the use of University of Wisconsin solution (Tsuchida et al., 2003).

Recent preservation experiments in porcine limbs using autologous blood in a hypothermic set-up with sub-physiological perfusion pressure have proven the technical feasibility and the great potential of this approach (Constantinescu et al., 2011). This is further supported by Müller at al. (2013), who were able to replant porcine front limbs after an extracorporeal perfusion time of twelve hours using a perfusate with added methylprednisolone with only minimal impact on ischemia/reperfusion injury as assessed by histopathology, markers of inflammation and endothelial cell activation.

### 3.2 Research models

*Udder*

The isolated perfused udder of various species, but predominantly ruminants, has been widely used in the study of milk synthesis and ejection and has also been used as a model for skin absorption in pharmacological studies (Tab. 4).

Use of the isolated cow's udder gland is largely based on a technique described by Peeters and Massart (1952). Here, specimens were perfused with normothermic oxygenated heparinized homologous blood for approximately two hours at constant pressure. Radioactively labelled substrates were added to the perfusate and their content in milk, venous return and tissue was recorded (James et al., 1956; Verbeke et al., 1959; Lauryssens et al., 1960). However, despite physiological $O_2$ consumption, milk yield declined rapidly after two hours (Lauryssens et al., 1961). To increase the flow rate, Hebb and Linzell (1951) included isolated perfused lungs in the circulatory system as the passage of blood through the lungs reduces its vasoconstrictive properties (Eichholtz and Verney,

**Tab. 4: Select perfusion models and their use in research**

| Organ | ECP LA suitable for | Findings | Model developed by |
|---|---|---|---|
| **Limb (equine)** | Modelling inflammatory responses in the context of laminitis | Endotoxin plays a role as causative agent for equine laminitis (Patan-Zugaj et al., 2012, 2014) | Patan et al., 2009; Gauff et al., 2013 |
| | Investigating the role of hyperinsulinemia in the context of laminitis | Hyperinsulinemia alters endothelin-1 expression in the equine laminar tissue which suggests endothelin receptor antagonists as a potential new class of agents in the treatment of laminitis (Gauff et al., 2013, 2014) | |
| | Pharmacokinetic studies in the context of joint disease | Acetylsalicylic acid and salicylic acid accumulate in the synovial fluid after systemic administration, despite subsiding systemic levels (Friebe et al., 2013b) | Friebe et al., 2013a |
| **Limb (porcine)** | Prolonged limb preservation for transplantation | Prolonged limb preservation barely influences ischemia/ reperfusion injury, ECP LA is a promising technique for use in transplantation surgery (Müller et al., 2013) | Constantinescu et al., 2011 |
| **Udder (caprine)** | Exploring physiological concepts (milk synthesis) | Metabolism of various substrates and their contribution to milk synthesis (Hardwick, 1965; Roets et al., 1979b, *inter alia*) | Hebb and Linzell, 1951; Hardwick and Linzell, 1960 |
| **Udder (bovine)** | Pharmacokinetic skin absorption studies | No cytotoxicity was recorded for various delivery systems, some formulations were superior regarding their maximum vitamin E delivery to deeper skin layers (Lampen et al., 2003) | Kietzmann et al., 1993 |
| | Pharmacokinetic studies in the context of mastitis (intra-mammary and systemic administration) | Cefquinome exceeds the $MIC_{90}$ values of common mastitis pathogens after a combined systemic and intra-mammary application (Ehinger et al., 2006) | |
| | Exploring physiological concepts (milk synthesis) | Metabolism of various substrates and their contribution to milk synthesis (James et al., 1956; Verbeke et al., 1959) | Peeters and Massart, 1952; Lauryssens et al., 1959 |
| **Uterus (bovine)** | Modelling inflammatory responses | The mucosal irritation potential of Lugol's iodine solution was shown on the Tyrode-perfused uterus. A hemoperfused uterus is a suitable model for inflammatory responses (Bäumer et al., 2002) | Kietzmann et al., 1993; Bäumer et al., 2002 |

1924; Newton, 1933). This technique was later replaced by the availability of artificial oxygenation devices (Hardwick and Linzell, 1960). Others included vasodilators (dibenamine or dibenzyline) to improve flow rates (Tindal, 1957).

With the intent to enhance the viability, udders were dissected from live animals under epidural anesthesia, which minimized the period between the interruption of blood supply and extracorporeal perfusion. Edema, defined as 20% or more weight gain, occurred in about half of the experiments and was associated with cessation of milk secretion within one hour. Nevertheless most secretory cells appeared normal in histology upon completion of the experiment (Hardwick and Linzell, 1960). Addition of an artificial kidney to the perfusion system allowed maintenance of high flow rates for a longer perfusion time (Hardwick et al., 1961, 1963; Hardwick, 1965, 1966; Linzell et al., 1967; Verbeke et al., 1968, 1972; Roets et al., 1974, 1979a,b).

In the 1990s, the isolated perfused bovine udder was used for pharmacokinetic research. Kietzmann et al. (1993) used it to find a treatment of mastitis. In these studies, warmed and oxygenated Tyrode solution was the perfusate of choice (Ehinger et al., 2006; Kietzmann et al., 2010).

The system's potential to study skin inflammation was recognized later. The collection of different parameters (glucose consumption, lactate production, lactate dehydrogenase activity, and pH in the perfusate, histological examination) indicated that skin viability was retained during a six hour perfusion interval and determination of udder skin-fold thickness demonstrated that no edema developed (Kietzmann et al., 1993). Subsequently, different anti-inflammatory drugs, administered systemically via the perfusate or topically to the skin, were tested in the model for their effect on eicosanoid synthesis (Bäumer and Kietzmann, 2001). The perfusion model also served as the basis for studies on the transdermal absorption potential of vitamin E acetate from cosmetic formulations (Lampen et al., 2003).

More recently, the isolated perfused bovine udder was used for preclinical trials on biodegradable magnesium implants (Schumacher et al., 2011).

*Heart*

The isolated perfused heart is an extensively used *ex vivo* whole organ research model. It has helped to gain a better understanding of cardiac physiology (contractile function, blood flow and metabolism) and pathophysiology (ischemia/reperfusion injury). The model forms the basis for the collection of viable cardiac myocytes or for the measurement of electrical activity (Bell et al., 2011). The technique dates back to Langendorff, who in 1897 introduced a model of retrograde perfusion of the isolated mammalian heart via the aorta, later known as the Langendorff heart. For this, the aortic root is slipped over a fixed cannula in the perfusion system, which is in turn connected to a reservoir containing a gassed and warmed perfusate, most commonly a modified Krebs-Henseleit solution. The model can either be used under constant flow or constant pressure conditions. It allows investigation of a broad spectrum of physiological, morphological, biochemical and pharmacological parameters and is generally accepted as a model to study drug-induced cardiotoxicity and ECG conductivity (Skrzypiec-Spring et al., 2007; Bell et al., 2011). The isolated heart can also be perfused in the working heart mode, i.e., the perfusate enters the left atrium via the pulmonary vein, flows into the left ventricle and onwards into the aorta (Chinchoy et al., 2000; Araki et al., 2005). The right side of the heart is not included in the latter system as for this a biventricular working heart set-up would be necessary, in which the right heart performs a physiological low pressure ejection (Demmy et al., 1992; Chinchoy et al., 2000)

*Uterus*

Another application of isolated organ perfusion is represented by the use of the bovine uterus for the study of mucosal inflammation (Tab. 4). For inflammation studies, a mixed Tyrode/homologous blood perfusate was used (ratio 1:4), whereas irritancy studies were carried out on only Tyrode perfused uteri. Both set-ups reported an average weight gain throughout the perfusion period of five hours of 20% (Bäumer et al., 2002; Braun and Kietzmann, 2004).

*Liver*

Chapman et al. (1961) described the first successful isolated liver perfusion in calves for up to 24 hours by cannulating the hepatic artery and portal vein simultaneously. This report demonstrated tissue viability based, amongst other things, on measurements of oxygen consumption, presence of constant blood flows and pressures, and only very slight changes in histologic parameters. The authors also postulated that the perfusate (autologous blood diluted with Krebs-Henseleit solution to a final hematocrit value of 25 to 35%) could be maintained free of bacteria for at least twelve hours of perfusion without the addition of antimicrobials (Chapman et al., 1961). A similar approach using homologous porcine blood with several additives (Tisusol, Rheomacrodex© 5% in dextrose, calcium glucoheptonate, heparin, xanthocholate) was successfully used to model hepatic metabolic clearance (Cameron et al., 1972). Following further improvements to the set-up and the addition of a dialysate circuit, autologous hemoperfusion of the isolated pig liver also produced good results and was shown to be a promising tool to study whole organ liver function and hepatotoxicity (Grosse-Siestrup et al., 2002b). In contrast to these results, Drapanas et al. (1966) reported on hemoperfusion of the isolated pig liver using heterologous (human) blood modified with low molecular weight dextran and heparin. After four hours of perfusion, this resulted in mild to moderate interstitial edema, reduced oxygen consumption and a sample weight gain of about 20%. As discussed above, half of this reported total weight gain occurred during the initial wash out period (20 to 30 minutes) with cold Ringer's solution, illustrating that this perfusate solution does not maintain oncotic pressure sufficiently (Drapanas et al., 1966). Human liver perfusion with Krebs bicarbonate buffer containing 20% prewashed human or bovine red blood cells, bovine serum albumin, α1-acid-glycoprotein, calcium and glucose, how-

ever, resulted in normal histological liver architecture after a perfusion period of approximately five hours. Nevertheless, the reservoir contained increased levels of alanine aminotransferase, likely indicating endothelial cell damage as a result of ischemic injury (Villeneuve et al., 1996).

*Intestine*

Few attempts to perfuse isolated large animal intestine have been made. For absorption studies, isolated porcine duodenum and ileum were perfused under normothermic conditions with Krebs-Ringer bicarbonate solution containing various additives including washed bovine or human erythrocytes, dextran T70, glucose, amino acids or cyclooxygenase inhibitors (Rehfeld et al., 1982; Messell et al., 1992; Hansen et al., 2000, 2004). The perfusion set-up by Messell et al. (1992) is based on an open system first developed for pancreas perfusion (Jensen et al., 1975). In this study, only perfusion pressure was continuously recorded, which was in turn associated with nerve function. No viability related data was reported, but stimulation by various means seemed to affect intrinsic nerves during the perfusion period of six hours (Messell et al., 1992). Hansen et al. (2000) applied the same model: No specific viability measurements were documented, but motor activity of the gut appeared normal with contractions leading to a short increase in perfusion pressure. To inform on intestinal function and pathophysiology in the context of ischemia/reperfusion injury, Polyak et al. (2008) developed a model of isolated equine large colon perfusion. A twelve hour perfusion period with a novel organ preservation solution and lacking any method of oxygenation proved superior to the use of oxygenated whole blood when arterial blood pressure, flow and intra-vascular resistance, electrolyte concentrations and mucosal integrity were assessed (Polyak et al., 2008).

*Kidney*

Efforts to establish and improve techniques for isolated kidney perfusion have been ongoing since 1903 (Pavy et al., 1903) with the main focus on the organ's functional preservation for subsequent transplantation surgery. First successful attempts were made using dog kidneys and normothermic perfusion conditions under physiological perfusion pressure in a closed system with a customized oxygenator supplying an $O_2/CO_2$ mixture. Two different blood preparations were used and weight gain was reported to be under 20% (Waugh and Kubo, 1969). Use of Tyrode or barium sulfate ($BaSO_4$) solutions for perfusion were associated with well-preserved histological morphology in experiments in which shockwave-induced lesions were investigated (Köhrmann et al., 1994). Perfusion with cell free Tyrode solution was on a par with autologous blood preparations in terms of reperfusion injury but was inferior in preserving renal function (Höchel et al., 2003).

*Lung*

The isolated perfused porcine and canine lungs have been described for physiological modelling. For this purpose either autologous whole blood (Bhattacharya and Staub, 1980) or Krebs solution augmented with Ficoll 70 as a colloid oncotic agent (Hellewell and Pearson, 1983) was used as the perfusate. Attempts to establish a set-up for prolonged lung preservation showed promising results after six hours of perfusion, although impairment of lung function occurred towards the end of the experiment. The perfusate of choice was blood diluted with Steen® solution (final haematocrit 15%), from which leukocytes and platelets had been eliminated (Erasmus et al., 2006). Stable lung function for up to twelve hours of perfusion with cell free Steen® solution was reported later (Cypel et al., 2008).

*Limbs*

The technique of isolated limb perfusion (ILP) serves a variety of research interests (Tab. 4).

In the context of equine laminitis an open ILP system using gassed Krebs-Henseleit solution was used to show that endothelium-derived nitric oxide modulates the response to vasoconstrictors and is therefore presumably an important regulator of blood flow in the equine digit (Berhane et al., 2006). In the same research field, Patan et al. (2009) demonstrated excellent tissue viability of the laminar tissue for a perfusion period of up to ten hours applying constant physiological blood pressures and using autologous whole blood as perfusate (Patan et al., 2009). The same group later showed the model's ability to respond to an inflammatory stimulus by adding lipopolysaccharide to the perfusate (Patan-Zugaj et al., 2012, 2014).

Another application of ILP is in pharmacokinetic studies, i.e., to assess drug distribution in the synovial fluid and synovial clearance after both intra-articular injection and systemic administration via the perfusate (Friebe et al., 2013a,b). In this context, the authors were able to show that following a perfusion period of eight hours with gassed Tyrode solution and sodium carboxymethyl cellulose, the intimal layer of the equine fetlock joint capsule appeared unchanged whereas the subintimal connective tissue presented mild signs of edema. In this study, four minimal criteria for an effective ILP of the equine distal digit were postulated: glucose utilization $\geq$ 200 mg/h, lactate production $\leq$ 400 mg/h, LDH activity $\leq$ 10 U/h, skin surface temperature $\geq$ 26°C (Friebe et al., 2013a). As described earlier, these criteria can only be used as reference for research under similar circumstances, as metabolic parameters are tissue and perfusion dependent (e.g., a closed system may lead to accumulation of lactate or the use of blood based perfusates may increase overall LDH levels due to the mechanical cell damage the pump inflicts on cellular blood components) and will also be influenced by the weight of the specimen and the perfusate volume.

Perfused isolated porcine limbs have furthermore been used for transdermal absorption studies due to similarities with human skin. This work included a comparison between low flow (100 ml/min) and high flow (230-250 ml/min, physiological) perfusions. The investigation suggested that a higher flow rate improved oxygen supply and reduced the risk of edema formation due to lower organ resistance (perfusion flow / perfusion pressure) and improved blood vessel integrity. An additional

comparison of two different perfusate solutions in the high flow group revealed improved hemodynamic parameters with use of a dialysate solution augmented with bovine serum and autologous erythrocytes over use of a whole blood dialysate mixture (Wagner et al., 2003).

## 4 Potential future applications and conclusion

Perfusion of isolated internal organs to preserve them for later transplantation is well established. Nevertheless, optimization of this process is ongoing in the field of extracorporeal perfusion research (Mancina et al., 2015). Successful preservation of severed limbs may increase the chances of reattachment. In this respect, ILP is a valuable technique for future research in the field of transplantation, replantation and prolonged preservation of limbs (Constantinescu et al., 2011; Müller et al., 2013).

The usefulness of ECP LA has been proven in many research areas and future applications may also include use of an isolated perfusion model to study cell specific migration towards different stimuli in the context of the respective donor organ. Controlled administration and tracking of specific labelled cell types, via *in situ*, *ex vivo* imaging methods, represent only a few of the possible advantages of this technique. Focusing on the migration of leukocytes would allow the use of ECP as a model for the early stages of inflammation, investigating, e.g., irritants or chemoattractants. Furthermore, if an inflammation-like condition can be produced, it would represent an interesting opportunity to investigate anti-inflammatory properties of new drugs applied via the perfusion fluid or topically (Friebe et al., 2013a,b). The authors are currently expanding this approach into the use of isolated perfused limbs to study short term events and interventions in the context of arthritis. Mesenchymal stem cells represent another interesting target cell. ECP LA could facilitate insights into migration and differentiation behavior of these cells in various tissues.

In veterinary research, recent efforts include, besides ILP, perfusion of intestine. With the future aim to study functional changes associated with colic (Polyak et al., 2008) and grass sickness (authors) in the equine patient. The behavior of equine chorionic girdle cells may also prove to be a fascinating field of study in this species.

To conclude, ECP of large animal specimens represents an excellent opportunity to answer research questions in organ preservation and transplantation, (patho)-physiology and pharmacology. This method may bridge the gap between basic and applied clinical research. Experiments can be carried out in a relatively physiological, standardized environment with large experimental numbers. By obtaining abattoir samples or surplus biological material from other experiments, this method strongly supports the 3R principles by reducing, refining and replacing live animal testing.

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## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgement

David Argyle is funded by the BBSRC.

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