Methodological review

Retrograde heart perfusion: The Langendorff technique of isolated heart perfusion

Robert M. Bell, Mihaela M. Mocanu, Derek M. Yellon *

The Hatter Cardiovascular Institute, University College London Hospital and Medical School, 67 Chenies Mews, London WC1E 6HX, UK

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ABSTRACT

In the late 19th century, a number of investigators were working on perfecting isolated heart model, but it was Oscar Langendorff who, in 1895, pioneered the isolated perfused mammalian heart. Since that time, the Langendorff preparation has evolved and provided a wealth of data underpinning our understanding of the fundamental physiology of the heart: its contractile function, coronary blood flow regulation and cardiac metabolism. In more recent times, the procedure has been used to probe pathophysiology of ischaemia/reperfusion and disease states, and with the dawn of molecular biology and genetic manipulation, the Langendorff perfused heart has remained a stalwart tool in the study of the impact upon the physiology of the heart by pharmacological inhibitors and targeted deletion or up-regulation of genes and their impact upon intracellular signalling and adaption to clinically relevant stressful stimuli. We present here the basic structure of the Langendorff system and the fundamental experimental rules which warrant a viable heart preparation. In addition, we discuss the use of the isolated retrograde perfused heart in the model of ischaemia-reperfusion injury ex-vivo, and its applicability to other areas of study. The Langendorff perfusion apparatus is highly adaptable and this is reflected not only in the procedure's longevity but also in the number of different applications to which it has been turned.

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* Corresponding author. Tel.: +44 207 380 9888; fax: +44 207 380 9505.
  E-mail address: d.yellon@ucl.ac.uk (D.M. Yellon).

1. Introduction

From the origins of isolated perfused heart preparations derived from the pioneering work by Carl Ludwig and his co-workers in the late 19th century through the development of the isolated frog heart by Elias Cyon in 1866 [1] to the later inception of retrograde mammalian heart perfusion by Oscar Langendorff in 1895 [2] (for more complete summaries of the historical development of the method, the reader is urged to read the excellent reviews of Zimmer [1] and Skrzypriec-Spring [3]), the isolated perfused heart model has proven invaluable in the development of our current understanding of heart physiology. From basic heart physiology in the form of myocardial function, through electrophysiology and the development of arrhythmia, coronary vascular function and regulation to the understanding of pathophysiology and its correlation to the clinical presentation of human disease states, such as hypertension, diabetes, heart failure and ischaemia/reperfusion injury, the Langendorff preparation has remained at the forefront of scientific endeavour. After over 100 years of study, the Langendorff preparation remains as relevant now as it ever has been: indeed with the development of methods of gene manipulation and protein expression, Langendorff perfusion remains at the forefront as a tool in dissecting the mechanisms of myocardial physiology and cellular signalling.

The aim of this manuscript is to describe the Langendorff method of isolated heart perfusion in the contemporary environment, the various applications of the technique, where problems are likely to arise and how to optimise the preparation to obtain reproducible and accurate data. We include information on the timings, solutions, stimulation settings that work well within our laboratory, but there are many variations on the theme, and investigators are urged to fully characterise their set ups and optimise conditions for their particular studies.

2. Principles of retrograde heart perfusion: the Langendorff method

2.1. The original description of the Langendorff method

According to Langendorff's original description, [2] a methodology that has remained fundamentally unchanged to this day, the heart is perfused by cannulating the aorta. With the perfusion buffer flowing retrogradely down the aorta, opposite to normal physiologic flow, the aortic valve is closed under pressure. With a column of perfusate within the aorta, the coronary arterial vasculature is thus filled via the two coronary ostia (left and right) in the respective sinuses of valsalva in the aortic root. The perfusion buffer then passes through the vascular bed, before being drawn off via the coronary veins to the coronary sinus in the right atria, located superior to the septal leaflet of the tricuspid valve on the posterior atrial wall. With free drainage of

the right atrium, the preparation can thus be maintained without any fluid filling of the ventricular chambers, which remain essentially "dry."

In the original description, the preparation was perfused under constant pressure, a mode of perfusion that remains common to this day, but there is also the option for constant flow perfusion, which offers particular advantages depending on experimental protocols that shall be discussed later. Coronary flow was estimated by Langendorff by collecting the effluent over a measured time period, and contractile function by measuring linear contraction of the preparation along its long axis, by passing a silk tie through the heart's apex. There are a number of alternate methods for measuring these parameters, but a number of groups continue to measure these basic physiological parameters in this way (and was our preferred methodology when we originally established murine Langendorff perfusion in the 1990s [4]).

The great advantages of this preparation are the reasons for the method's longevity: simplicity of the preparation, low cost, reproducibility and the ability to study the organ in isolation of other organ systems and exocrine control that may confound physiological measurement. In the context of our work for example, we can contemplate ischaemia/reperfusion protocols that would prove lethal to the whole animal if the same experiment were performed in vivo, and similarly pharmacological inhibitors of a particular signalling pathway used without fear of impacting upon non-cardiac organ function or triggering stress response and inducing potentially confounding neuro-humoral compensation.

There are, inevitably, limitations to consider too; the isolation from the whole animal takes any study further away from clinical relevance. Furthermore, the Langendorff preparation, which may well be viable over several hours, must nonetheless be considered as a dying preparation. We have found from our own experience work the same as Sutherland and Hearse have published—approximately a 5–10% per hour deterioration in contractile and chronotropic function [5]. However, within these limitations, Langendorff perfusion studies can be well designed and generate useful and insightful data.

The final advantage of Langendorff perfusion is its wide species applicability. A wide range of mammalian species have been studied in this way, ranging from mice, through rat and rabbit, to larger animals such as dog and pig, to primates and even human heart [6,7]. Thus this flexible and essentially simple method of study of myocardial physiology has been both widely adopted and widely adapted for particular investigative requirements.

2.2. Modes of perfusion: constant pressure versus constant flow

There are two modes of retrograde heart perfusion: constant pressure versus constant flow. The former can be readily and cheaply achieved by maintaining a constant hydrostatic pressure through a set

height column of fluid—such as positioning a reservoir and its fluid meniscus a known distance above the tip of the perfusion cannula in the heart preparation's aorta. This represents possibly the cheapest and simplest manifestation of a Langendorff perfusion apparatus, but certainly is not the only way in which a constant pressure can be achieved. Indeed in Langendorff's original work, constant perfusion pressure was attained and monitored via a sealed pressurised chamber and a connected manometer. Like modern negative feedback pressure control loop peristaltic pump systems, Langendorff's apparatus had the advantage of a smaller perfusate volume required to complete the circuit when compared to a gravity-fed fluid-column system, which is an important consideration where blood, red blood cell augmented perfusion buffers or perfusion buffers that include a study drug are to be used by virtue of the limited availability or expense of the resource.

The aforementioned pressure control circuit using peristaltic roller pumps are well characterised [8] and commercially available—and offer the advantage of being able to switch from constant pressure to constant flow seamlessly. A further advantage of such pump-control systems is the continuous measurement of coronary flow, through calibration of the pump speed with known standards of coronary flow rate, which avoids the use of potentially expensive flow-meters. Constant pressure mode perfusion is ideal where reliance upon the heart's auto-regulation of coronary tone is required, and particularly where there is ligation of part of the perfusion bed, as occurs in models of regional myocardial ischaemia—a situation where constant flow systems would administer far more coronary perfusate per unit volume of available myocardium than before coronary ligation, with the risk of resulting coronary artery damage through shear stress.

Constant flow preparations, where coronary flow is administered by a peristaltic pump, are of particular use when studying coronary vascular tone/smooth muscle/endothelial function. Measurement of perfusion pressure is relatively straightforward using a pressure transducer in the fluid circuit ahead of the perfusion cannula, and from which, with a known flow rate, coronary vascular resistance can be calculated using a derivation of Ohm's law. Thus vasoactive substances can be directly studied in whole heart preparations.

2.3. Measuring physiological parameters

Measurement of physiological end points can be achieved using straightforward techniques, but the investigator can be presented with particular technical challenges when it comes to implementing them in practice.

2.3.1. Coronary flow

With respect to coronary flow, methods for measurement have already been alluded to in the previous section, and will depend on the design of the apparatus employed. In the case of peristaltic pressure control systems, the signal voltage that alters the pump speed can provide a calibrated real-time measure of coronary flow rate, which in more simple preparations, such as gravity feed orthostatic pressure apparatus would have to be determined either through the use of a flow meter or measurement of effluent volume over a measured period of time, the latter of which has the greatest opportunity for inaccuracy.

2.3.2. Myocardial contractility/left ventricular systolic and diastolic function

Measurement of myocardial contractility can either be via linear force contraction with a tie through the apex and connection to an isometric force transducer (as in Langendorff's original studies) or through the insertion of a left ventricular balloon, accessed by removing the left atrial appendage, and passing the balloon through the mitral valve. This technique becomes increasingly challenging the smaller the hearts used, but have been successfully employed in mouse [9] and neonatal rat heart [10,11]. The ideal characteristics of a

left ventricular balloon are well described, needing to be made of a material that is highly compliant and ideally should be as thin as possible (pre-stretched cellophane is one good practical material for balloon construction), have no elasticity, and a frequency response curve that neither damps nor amplifies the recorded signal at the range of heart rates likely to be encountered [9,12]. The volume of the balloon should be such that it is able to fill the ventricular cavity; too small and there will be failure to properly estimate ventricular function, too large and the potential for inducing endocardial necrosis once a pre-load diastolic pressure is set. The balloon is mounted on a length of non-compliant tubing (or in the case of mouse heart perfusion, typically on a 21 gauge non-bevelled needle) and connected via a fluid link to a calibrated pressure transducer. Great care needs to be taken to ensure that no air is to be found in the circuit; air unlike water is compliant and will therefore dampen the signal. Once calibrated, the left ventricular balloon can be used to measure a variety of contractile functional data, including heart rate, contractile function, end diastolic pressure, and derivatives such as dP/dT maxima and minima. Moreover, by adjusting the volume of the balloon, ventricular stretch can be assessed, and Frank–Starling curves established, which can be of particular value in many functional experiments.

2.3.3. Temperature

As indicated in Section 2.4.1, temperature monitoring and regulation is crucial part of maintaining a Langendorff preparation and the subsequent interpretation of data obtained. Typically this involves the insertion of a thermocouple into the heart, usually into the right ventricle. Strict monitoring and regulation of temperature (the set point for which will vary according to the body temperature of the species being studied) can then be achieved.

2.3.4. Electrocardiogram (ECG)

Monitoring of the electrocardiogram or monophasic action potentials can be readily achieved on the isolated perfused heart through the use of appropriate electrodes. Assuming the selection of an appropriate species of study, investigators may use the Langendorff preparation in the investigation of arrhythmogenesis and its applicability to human disease [13].

2.4. Perfusate

2.4.1. Krebs Henseleit Buffer

By far the commonest bicarbonate buffer used by researchers utilising the Langendorff perfused isolated mammalian heart is the buffer described by Krebs and Henseleit in 1932 [14]. When bubbled with 5% CO_2, Krebs–Henseleit buffer (KHB), composed of NaCl 118.5 mM, NaHCO$_3$ 25.0 mM, KCl 4.7 mM, MgSO$_4$ 1.2 mM, KH$_2$PO$_4$ 1.2 mM, glucose 11 mM and CaCl$_2$ of 2.5 mM, will possess a pH of 7.4 at 37 °C. However, as originally formulated, the content of calcium chloride at 2.5 mM was well in excess of physiologic bioavailable ionised calcium in whole blood (around 1.3 mM in rat heart for example [15]), an error likely to be related to the failure to take into account calcium binding to circulating protein. Once this oversight was recognised, most researchers have reduced the calcium concentration in their modified Krebs–Henseleit perfusion buffer to better reflect physiologic calcium, with a range between 1.2 and 1.8 mM [5]. Thus formulated, studies using KHB perfused hearts are widely published, but there are further modifications that can be undertaken to the basic compositions, particularly if heart metabolism is to be studied. The use of glucose as the metabolic substrate in KHB is perhaps something of a surprise given the preference of the normoxic myocardium towards free fatty acids, [16] but relies on the ability of the heart to successfully and efficiently extract almost any metabolic substrate as an energy source [5,17]. Addition of pyruvate or free fatty acids are certainly possible, but the latter presents particular problems when traditional sintered glass oxygenators are used to

bubble the buffer; significant frothing can occur. The other problem with the glucose in KHB is the concentration, which is very high—and within what could be regarded as the "diabetic range" (11 mmol/l, near double that of the 5–6 mmol/l in a normal animal). With an increasing interest in disease states and their impact upon physiological and pathophysiological performance of the heart, this high level of glucose needs consideration; the basis for a higher than physiologic glucose is probably derived from the aforementioned lack of an alternate energy substrate in the perfusion buffer, which is thus compensated for by the use of more glucose. We have found that it is possible to perfuse rat hearts with 5 mmol/l without significant haemodynamic impact or consequence upon ischaemia/reperfusion protocols (unpublished data), but it may be preferable to supplant the excess glucose with an alternate substrate such as pyruvate or free fatty acid in a fully characterized model.

A further limitation of KHB is the limited oxygen-carrying capacity of crystalline solution, and the low oncotic pressure of KHB [17]. Indeed, compared to blood-perfused isolated hearts, there is significant accumulation of total tissue water, indicative of oedema, particularly following ischaemia/reperfusion protocols [15,18]. Moreover, blood perfusion of isolated hearts are associated with better preservation of left ventricular function, both over a steady state (with less than 5% per hour deterioration of contractile function [5]) and following ischaemia/reperfusion injury [15,18]. However, despite limitations, KHB remains a practical and useful method of maintaining an isolated perfused heart for many hours; depending on the study design and parameters being studied, alternative perfusates may need to be considered.

2.4.2. Whole blood perfusion

While substantially more complex and technically challenging than KHB perfused Langendorff perfusion, blood perfused isolated heart preparations are a practical proposition with the isolated hearts: hearts so perfused demonstrate comparatively minor perturbations in metabolism, yet possess robust functional parameters [19]. In essence, the method calls upon the use of a donor animal to be anaesthetised, and large artery and vein cannulated. The arterial line is then connected to the isolated heart's aortic cannula, and the effluent from the heart collected, and pumped back into the donor animal's venous system whereby it will be re-oxygenated by the donor. Essentially, the donor animal replaces the crystalloid Langendorff's reservoirs. The perfusion can either be constant pressure or constant flow, and the return blood is filtered (200 μm) to remove particulate contamination. While almost certainly more physiologic than the KHB perfused heart, not least in the much lower rates of coronary flows (2.0 ± 0.3 ml/min/g wet weight heart in rat, a tenth of what would have been seen with crystalloid perfusion [18]), there are nonetheless disadvantages with this methodology—not least in terms of on-going haemolysis as the donor blood passes through the extracorporeal circuit. Moreover, there is the potential confounding influence of the donor animal's humoral response to a not insignificant proportion of its circulating volume being diverted to perfuse the isolated study heart: a significant increase in endorphin release is recognised [19]. In most part, this Langendorff methodology has been largely supplanted by in-vivo studies in investigations into ischaemia/reperfusion injury, and studies of metabolism are often better served in working heart preparations with substrate fortified crystalloid solutions.

2.4.3. Red blood cell augmented crystalloid buffers

Red blood cells, usually harvested from bovine blood, can be spun down to remove white cell and platelet components of the blood, and added to a crystalline buffer, mixed with dextran/albumin to obtain a near normal osmolality and oncotic pressure [3,5]. When oxygenated using a dedicated oxygenator, the red-cell augmented crystalline buffer can be used to perfuse the heart in the similar way to KHB perfused heart, the difference being that the red blood cells are recycled. As with donor blood perfused hearts, the preparation is extremely stable (<5% myocardial functional loss per hour), physio-

logic coronary flow rates, and the hearts suffer significantly less oedema [5]. However, haemolysis remains a problem. Immunogenicity of the preparation—the use of bovine cells in rat heart—is thought to be minimal due to white cell and protein depletion during red cell purification [5]. However, given the additional complexity and expense of this preparation, KHB has retained sway within the perfusionist community; those seeking more physiologic conditions are now more likely to perform their experiments in-vivo rather than in the ex-vivo model.

2.5. Potential pitfalls

When setting up a Langendorff system for the first time, it is useful to consider some of the common problems that can lead to either failure of the preparation or to data that may not be readily interpreted or difficult to repeat. Identification of these potential pitfalls can then be reflected in the design of the apparatus and experimental protocols undertaken to optimise the value of results thus obtained.

2.5.1. Temperature

As mentioned earlier, the maintenance of temperature throughout the Langendorff experiment is critical. Variations of temperature impact upon myocardial contractility and heart rate in the steady state, [5,9,20] has profound bearing upon experimental end points such as myocardial viability following ischaemia/reperfusion injury, [21–24] and transient hypothermia to 26 °C [25] or hyperthermia to 42 °C [26] prior to exposure to injurious ischaemia has been shown to trigger preconditioning-like cardiac protection. Moreover, the heart has a high surface area for its volume—a problem exacerbated in the smaller animal species—and thus a significant potential for radiant heat loss and consequent hypothermia, an issue that inevitably does not arise in vivo for a heart encased within the pericardium in a closed thoracic cavity.

In Langendorff preparations, the main sources of exogenous heat are either from pre-warmed perfusion buffer flowing through the coronary vascular bed, submersion of the suspended heart in a suitable organ bath or exposure to a radiant external heat source. Perfusion-independent sources of heat become increasingly important in models where coronary flow is attenuated (in, for example, regional ischaemia), or indeed stopped completely (as in global myocardial ischaemia models). Even in non-ischaemia models, coronary flow alters with time over the course of an experimental protocol, leading to changes in myocardial temperature; temperature of the myocardium is inversely proportional to the coronary flow, an observation that becomes more evident in the smaller animal species such as mouse with radiant heat loss from its high surface area to volume ratio. Therefore it is essential that temperature of the myocardium is continually monitored and altered accordingly.

2.5.2. Perfusion pressure and flow restrictions

When building a Langendorff perfusion rig for the first time it is essential to know whether the pressure of the perfusion buffer reaching the heart is what is anticipated. The perfusion pressure depends on the species under study, typically ranging between 70 and 80 mm Hg, and resulting in coronary flow rates largely dependent upon the size of the hearts under study (typical figures for mouse, rat and rabbit being 2 ml/min, 20 ml/min and 40 ml/min respectively). Therefore the delivery system has to be capable to maintaining the desired pressure at least over the range of the anticipated inclusion criteria for coronary flow for the species studied. It may seem logical that if a column of perfusate is a certain height above the tip of the perfusion cannula, then the pressure seen at the cannula should be the same—and while this is true of a static fluid column, once there is flow, any restriction in the delivery circuit can lead to a significant drop in the perfusion pressure. Therefore it is prudent to measure the

perfusion pressure at the cannula over the anticipated range of coronary flow. Potential restrictions to flow may exist in the diameter of tubing used, coil heat exchangers, tubing connectors, sintered glass in-line filters and even at the cannula itself.

2.5.3. Maintenance of the perfusion circuit

One of the commonest reasons for experimental failures on an established Langendorff rig is microbial infection. Endotoxin release from microbes can significantly alter the performance of the isolated heart and adversely affect experimental outcomes. The risk of microbial overgrowth can be minimised in the design and construction stage; perfusion circuit should be as continuous as possible with as few tubing connections, step changes in tubing diameter or side branches where stagnant fluid may harbour microbes must be kept to an absolute minimum. Thereafter, good cleaning practice is recommended. Thorough flushing of the Langendorff apparatus after use to remove bacterial substrates such as glucose from the plastic and glassware is advocated following each day's experiments, and it is our standard practice to used boiled water at over 80 °C to minimise bacterial contamination. Thereafter, a deeper clean every 2 weeks are typically employed, using either the commercially available detergent, Decon 90, or 10% hydrochloric acid. The latter particularly being useful in removing any persisting salt deposition within the perfusion apparatus. The system is then flushed repeatedly with capacious amounts of de-ionised water. In the case of commercially available systems, the manufacturer will provide cleaning instructions. Note that some organic molecules (e.g. insulin) adhere strongly to the walls of the reservoirs and tubing and require special care to remove them from the system.

3. Design and construction of a Langendorff rig

3.1. Cannula design and specification

The aortic cannulae can be made of plastic, glass or metal of a diameter appropriate to the size of the aorta. As may be predicted, there are competing demands being placed on the design of the cannula, which needs to fit inside the aorta (too large, and the aorta may tear during cannulation), but be of sufficiently large diameter as to not represent a significant flow restriction (and therefore cause a pressure drop) at the rates of coronary flow anticipated during the experiment. For mouse heart, a 21-gauge stainless steel leur-lock needle is often employed, with the bevel removed and grooves machined circumferentially so that the aortic ties can grip the aorta to the cannula and thus avoid accidental decannulation. Similar cannulas are constructed for rat (3 mm outer diameter) and rabbit (4 mm outer diameter), again with distal grooves machined. Ideally, these cannulae should be a part of the temperature control circuit and heated, as described by Sutherland and Hearse [5].

3.2. Temperature monitoring and control

As indicated in Section 2.4.1, adequate temperature control is essential for isolated heart perfusion: the whole perfusion circuit should therefore be maintained at a constant temperature. One approach may be to enclose the Langendorff apparatus within a sealed box, suffused with heated humidified air at 37 °C. However, this is rarely done due to the practical limitations of such an approach, and thus the majority of investigators chose to use water-jacketed reservoirs and heat exchangers to maintain the perfusion buffer temperature at 37 °C. Ideally, all interconnecting tubing should also be warmed in a similar fashion by water fed from a thermostatically controlled high flow output pump, and include also the cannula itself. However, some heat loss is inevitable, particularly with the lower coronary flow-rate animals—but the temptation to raise significantly the water jacket temperature should be resisted; significant over-

heating of the heart may occur when there is an increase in coronary flow (as may occur following regional ischaemia when the heart is reperfused), with consequent suppression of myocardial function [5].

Maintenance of normothermia is further aided by emersion of the perfused heart in a water-jacketed organ bath filled with perfusion buffer, which is ideal in studies that do not require the collection of the heart's effluent. If the latter is required, then enclosure within a heated chamber, with sealing over the aperture of the camber (a challenge in an instrumented Langendorff heart) represents an alternative for maintaining good temperature control.

3.3. Choice of perfusate and oxygenation

As discussed in Section 2.3 there is more than one choice of perfusion buffer available for those undertaking Langendorff perfusion, and the choice ultimately made by the investigator to fit with the parameters that are due to be measured. KHB is a good choice for a wide range of studies, and represents the cheapest and most convenient method of perfusing the isolated heart. Although the oxygen carriage is limited, oxygenation of this bicarbonate buffered solution can be simply achieved through bubbling 95% O_2/ 5% CO_2 through sintered glass oxygenators. However, the use of buffers containing proteins or red cells (that can be damaged through frothing of the solution) requires a different approach to oxygenation, potentially using membrane-type oxygenators, [27] thin-film oxygenators [28] or any other method of oxygenation that does not result in large bubble formation, all of which have been used successfully for these types of solutions. Regular sampling of the perfusion buffer via a blood gas analyser enables the investigator to monitor not only oxygenation but pH to ensure that experimental parameters remain consistent.

3.4. Constant pressure apparatus

Where budgets are constrained, it is perfectly feasible to construct a simple and effective constant-pressure apparatus using widely available off-the-shelf glassware and tubing, as long as the apparatus has flow characteristics sufficient to deliver adequate buffer flow to meet the needs of the heart under study. Using no more than gravity, a set column height of perfusion, with water jacketed reservoirs can be a highly effective piece of apparatus. However, such an apparatus will, through necessity, have a significant dead-space volume. This can represent a problem where it comes to adding in drugs, tracers or antibodies; the need to make more solution to fill the dead-space will rapidly increase the cost of these consumables and ultimately lead to more wastage. Moreover, this type of perfusion apparatus will not be able to provide the option of constant flow.

The alternatives are commercially available pump-controlled systems, where perfusion pressure measured above the cannula can be used as a signal in a negative feedback loop to control a peristaltic pump. Such equipment offers the advantage of real-time coronary flow monitoring and the ability to switch seamlessly to constant flow with perfusion pressure monitoring. Moreover, the circuit can be designed with a much smaller dead-space with material and cost-saving potential. The major difference between a pump and gravity feed system is the necessity of installing a compliance chamber to the pump-feed system to prevent high-frequency oscillations from the peristaltic pump damaging the aortic valve apparatus (Fig. 1).

3.5. Constant flow apparatus

The constant flow Langendorff apparatus can be assembled in essentially the same fashion as the pump-controlled constant pressure Langendorff system: a peristaltic pump feeding into a compliance chamber, to which the aortic cannula is attached. A pressure transducer connected above the cannula is required to monitor the coronary perfusion pressure through the study (Fig. 1).
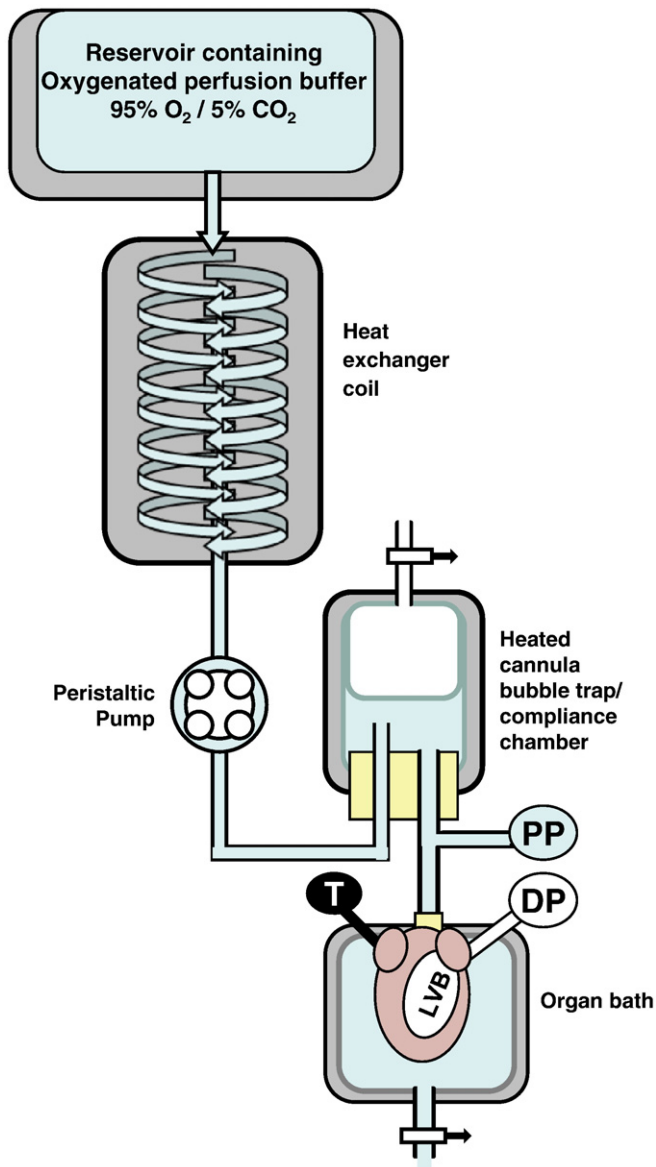
Fig. 1. Simplified scheme of a Langendorff system in constant pressure or constant flow. The heart is cannulated via the aorta and retrogradely perfused, and suspended within a warmed organ bath/chamber. Temperature is monitored using a themocouple (T) inserted in the right ventricle. A side arm to the perfusion cannula is ideal for measuring perfusion pressure (PP) with a pressure transducer, but may also be used for administering drugs/dyes. A left ventricular balloon (LVP) is inserted into the left ventricle, from which left ventricular developed pressure (DP) can be measured by pressure transducer. A peristaltic pump may be used to administer constant flow, or with a negative feedback circuit using measured PP, to maintain constant pressure. Alternate methods of maintaining constant pressure perfusion are discussed in the text. All pipework and chambers are contained within water jackets where possible, to maintain perfusion buffer temperature at 37 °C.

3.6. Ventricular pacing

A denervated, isolated perfused heart that is no longer under neuro-humoral control will not beat at the same rate as the heart in vivo. Moreover, the sinoatrial (SA) node is not supplied by the coronary vasculature, but rather from extra-cardiac vessels severed when the heart is extracted from the thoracic cavity, [5] it is not particularly surprising that the preparation is comparatively brady-cardic when compared to in-vivo: the isolated perfused mouse heart will have a rate of 380–420 beats per minute compared to 580–600 in-vivo, and a rat 250–320 compared to 350–400. The longer RR interval may alter calcium homeostasis, with consequent alterations in

contractile function, [9,29] and further changes may be expected following ischaemia/reperfusion. Therefore ventricular pacing is capable of removing heart rate as a variable when comparing experiments, and may avoid the potential for bradycardia related arrhythmia [5], and can be achieved relatively simply by using the cannula as one electrode, and a silver wire or stainless steel needle as the other in the epicardium of the right ventricle. In our laboratory for example, mouse heart pacing is at 600 bpm, using a square form wave of 4.0 ms duration and voltage amplitude of $1.5 \times$ threshold.

4. Langendorff heart preparation

4.1. Harvesting the heart

Any surgical intervention upon an animal will require the induction of a general anaesthesia. While there is no ideal anaesthetic available (for example, inhaled volatile anaesthetics are both cardioprotective [30,31], an important consideration in studies where alternate cardioprotective regimen may be the subject of study, and also require the use of expensive anaesthetic equipment with gas scavenging facilities), barbiturates are the usual preferred method of inducing a deep anaesthesia in animals. Barbiturates have narrow therapeutic window: the onset of deep anaesthesia is very close to the onset of cardiorespiratory suppression, but this is a problem that can be circumnavigated. Used with caution yet with minimal equipment costs, a single injection of barbiturate (for example, pentobarbitone 60 mg/kg i.p.) can be employed with good effect. Many groups, including our own, also take the opportunity to co-administer an anticoagulant such as heparin (100 I.U.), which reduces the theoretical risk of thrombus formation within the coronary vasculature or ventricular chambers. An alternative to anaesthesia may be the rapid cervical dislocation but this is regarded as undesirable due to the potential activation of sympathetic tone. Ultimately, the anaesthetic method of choice is dictated by local animal welfare requirements.

Once the onset of a deep anaesthesia has been established through, for example, the disappearance of the pedal pain withdrawal reflex, it is then possible to proceed to the surgical harvesting of the heart. There are many minor variations to this technique, particularly when it comes to comparing different species, but in principle involves the same process of thoracotomy. A skin incision is performed at the xyphoid-sternum and continued to the lateral ends of left and right costal margins. The incision is then continued through the ribs at the left and right anterior axillary lines to create a clamshell thoracotomy. The anterior chest wall is deflected upwards and the pericardium is opened, which gives a good access to the great vessels. The heart is then removed by transecting the descending aorta and inferior vena cava, followed by the ascending aorta and superior vena cava and then transferred to a dissection dish containing ice cold Krebs–Henseleit buffer. It is to be expected that in the initial experiments a variable proportion of extra cardiac tissue will be removed as well, but this will improve with experience. At this stage of the experiment the priority is to transfer the heart into the cold buffer as quickly as possible to avoid any detrimental effects of hypoxia.

4.2. Cannulating the aorta

For adult rat and larger species, the hearts are transferred directly from the ice cold buffer onto the cannula which is already in place in the perfusion system and through which perfusate is dripping, enabling fluid to fluid connection and minimizing the risk of air being introduced into the coronary vasculature producing an air emboli, with consequent microvascular obstruction and detrimental impact upon function and experimental outcomes. The heart can then be temporarily secured by the aorta to the cannula with a bulldog clip, and then secured with 2 sutures, followed by trimming of the excess extra cardiac tissue.

For smaller animals and in particular the mouse, in which the aorta is of both small in caliber and friable, the aortic cannulation is better performed in the cold buffer dish under direct illuminated and magnified visualization. After trimming of the excess extra cardiac tissues, the ascending aorta is exposed and cannulated with a primed 21 gauge stainless steel murine cannula (Fig. 2) under Krebs solution to avoid air embolisation of the coronaries. The cannula is inserted into the aorta by maneuvering the heart with as little trauma as possible. This is best achieved using two small forceps to hold the aorta gently open and slowly sliding the aorta onto the cannula with gentle left to right movements. On transferring the cannula to the perfusion rig, the perfusate should be allowed to run through the system so that a fluid to fluid connection can be rapidly established.

Of special note, in all species care should be taken not to insert the cannula too deeply into the aorta as this can lead to mechanical rupture of the aortic valve leaflets, with consequential gross aortic regurgitation. A competent aortic valve is essential to maintain perfusion pressure at the coronary ostia, and in its absence, reduced coronary perfusion can be anticipated as buffer will be lost via the left ventricle, particularly once the mitral valve is rendered incompetent by the insertion of a ventricular balloon. Consequently, the heart will not perform as expected, and should be excluded from further study. At the other extreme, if the cannula is secured above the brachiocephalic artery, buffer will be lost through free drainage with the inevitable loss in coronary perfusion pressure.

Once the cannulated heart is safely established on the Langendorff perfusion circuit, the flow will stabilize for the given perfusion pressure and the heart should start to beat vigorously within seconds. The time taken from the moment of the opening of the thorax until the heart is mounted and perfused on the Langendorff system needs to be well under 5 min to avoid either the potential effect of ischaemic
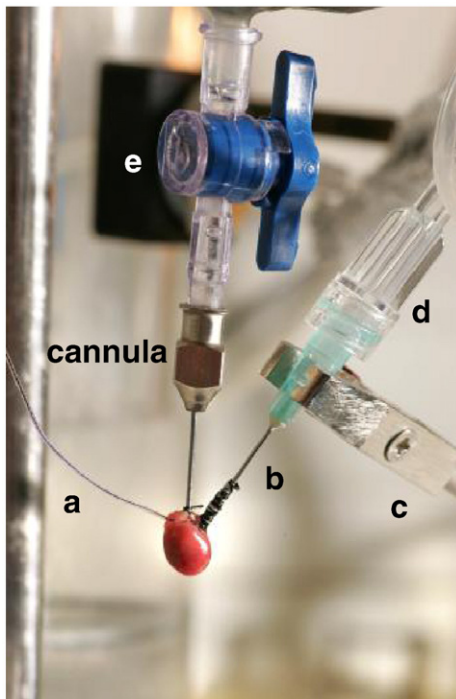

Fig. 2. A Langendorff-perfused mouse heart. During the perfusion protocol, the heart would normally be immersed in an organ bath—not shown here for photographic purposes. Figure key: (a) thermal couple for continual temperature monitoring; (b) intraventricular balloon inserted in the left ventricle and mounted on a 21-gauge non-bevelled needle; (c) a micromanipulator which helps placing and keeping the balloon in the left ventricle; (d) one end of the non pliable tubing that provides the hydraulic line to a pressure transducer mounted at the same height as the heart; (e) a 2 way tap placed between the bubble trap and the cannula, which serves to switch off the buffer during global ischaemia.

preconditioning due to delayed perfusion [32,33] or damaging the heart if the hypoxic duration is too long. In large animals, where the heart can be transferred directly to the perfusion apparatus, this can usually be achieved within 30 seconds, and on smaller animals such as mouse, within 120 seconds.

4.3. Instrumenting the heart

A small incision is made in the right ventricular outflow tract/pulmonary artery to allow free drainage of coronary effluent. Through this orifice a fine thermocouple can be retrogradely passed into the right ventricle and attached to a digital thermometer to permanently monitor the temperature inside the heart. A computerised system will use this information to adjust the temperature of water in the heated chambers, but, if this facility is not available, the temperature provided by the thermostated pump can be manually modified, or if coronary effluent does not need to be collected for analysis, the level of emersion in a warm buffer filled organ bath adjusted.

Heart rate and the left ventricular developed pressure are measured using an intra-ventricular balloon. The deflated balloon should be inserted gently in the left ventricle either via the left atrium, after removing the atrial appendage (Figs. 2 and 3A), or via the pulmonary veins. Once positioned in the heart, the balloon is inflated to 5–10 mm Hg in order to pre-load/stretch myocardial fibres to obtain an optimal developed systolic isovolumaemic pressure. The size of the balloon in relation to that of the heart is an important factor in obtaining a good signal. The coronary flow rate, the temperature and the pressure signals can be displayed, monitored and recorded, with the information used later for further calculations.

Key points:

- The heart is a delicate organ and must be handled gently to ensure reproducible results [9,34].
- The time delay between harvesting the heart and being completely perfused with buffer needs to be kept under 5 min to avoid damage or preconditioning [33].
- Inserting and positioning of the cannula require special attention.
- Air bubbles in the system could kill the heart or deleteriously affect its function.
- Maintaining the heart at a constant temperature throughout the experiment is essential and may be challenging when using small hearts or in studies in which coronary flow is temporarily modified (e.g. vasoconstriction, vasodilation, ischaemia).

4.4. Exclusion criteria

Inclusion and exclusion criteria are useful in the pursuit of consistent and reproducible data. Prolonged time to cannulation can lead to either myocardial damage or sufficient activation of stress signalling as to render the heart preconditioned [32,33]. Very high flow rates may be indicative of a tear in the aortic wall, or an incompetent aortic valve—in either case, the observed flow may not be representative of the buffer flow through the coronary system, and it may be difficult to know the perfusion pressure in the coronary tree. Similarly, low flow rates could be indicative of a cannula that has past and partially occluding the coronary ostia, thus presenting an obstruction to coronary flow, or represent an embolisation, be that air or particulate, obstructing the vascular tree. Highly arrythmogenic hearts and hearts that fail to reach a reasonable level of contractile function are also suggestive of a heart preparation that is not well perfused—and it is well to consider exclusion criteria that may enable elimination from analysis of such hearts where, through a technicality, a problem has occurred that will confound the experimental data. It is good practice that these should be clearly defined ahead of commencing the experimental study, and examples of exclusion criteria for a range of mammalian species are included in Table 1.
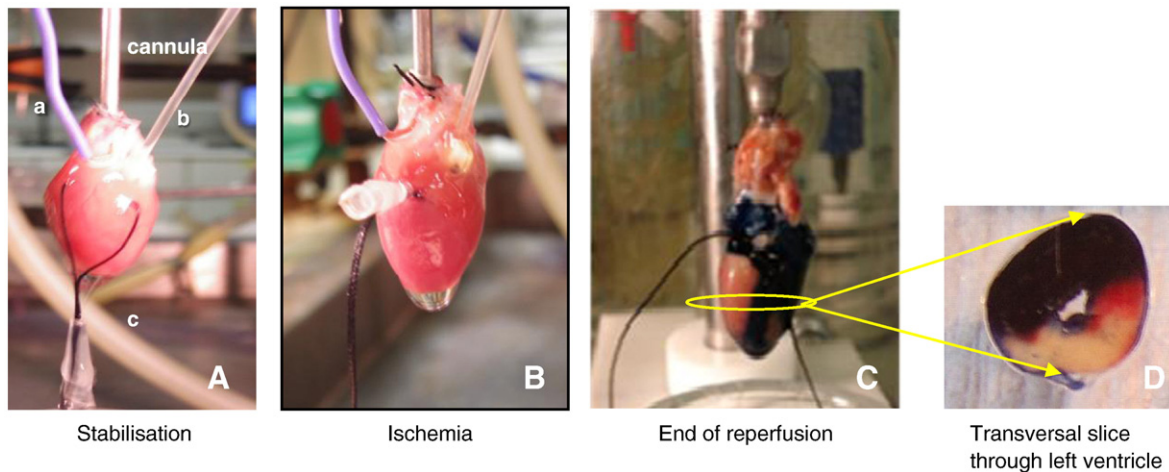
Fig. 3. Rat heart mounted in the Langendorff system. In stabilisation (3A) the suture is put in place around the left anterior descending artery (LAD), and formed to make a snare. During ischaemia (3B), the snare is tightened around LAD and secured in place by 2 plastic pipette tips which are pushed one into the other as an interference fit, maintaining the snare's tension during ischaemia. This temporary ligation is released during reperfusion but in the end of this period (3C), a secure knot is tied and Evans blue is perfused via the aortic cannula, staining the non risk zone dark blue. A TTC stained rat heart slice (3D), showing the non risk zone dark blue, and the risk zone formed by the viable myocardium (pink) and the dead (infarcted) myocardium (pale yellow).

5. Specific applications

5.1. Ischaemia/reperfusion injury

The Langendorff heart preparation, which initially offered valuable information regarding heart physiology, is now used in a multitude of biochemical, metabolic, pathophysiologic and pharmacological studies. One of the main fields in which these studies conjoin is cardioprotection against ischaemia–reperfusion induced cell death. In the following subsection the experimental protocol of inducing and assessing ischaemia–reperfusion injury is described, using infarct size as the end point.

5.1.1. Experimental protocol

5.1.1.1. Stabilisation.
After commencing retrograde aortic perfusion, a good heart preparation will attain a stable steady-state within 15 min–20 min. During this period, hearts must meet certain functional requirements that are set out in pre-determined exclusion criteria (summarized in Section 4.4). Once an experiment has been shown to be functioning within acceptable parameters, it is then

Table 1
Summary of exclusion criteria for Langendorff perfused heart.

Parameter	Mouse	Rat	Rabbit
Time to perfusion (min)	>4	>3	>3
Coronary flow (ml/min)	<2.0 or >5.5	<10 or >28	<55 or >80
Arrhythmia duration (min)	>3	>3	>3
Heart rate (beats per min)	<320 or >620	<70 or >400	<150 or >190
Left ventricular developed pressure (mm Hg)[a]	<60 or >140	<70 or >130	<70 or >130

A summary of exclusion criteria applied to Langendorff perfused hearts. Prolonged cannulation times can result in either deleterious injury to the isolated heart, or induce stress pathways that may confound results. Therefore rapid cannulation is preferred without jeopardising the preparation. At the end of the stabilisation period (typically around 20 min of normoxic, normothermic perfusion), very low or high coronary flow rates may be indicative of problematic perfusion (see text), and prolonged arrhythmia, bradycardia and poor left ventricular developed pressure too are all signs of poor myocardial function secondary to a problem in establishing rapid effective perfusion, and thus should be excluded from further analysis. Exclusion criteria should be established prior to commencing study, and those experiments that fail clearly documented.

[a] Left ventricular isovolaemic pressure is measured with a pre-set pre-load of approximately 10 mm Hg to optimise the observed LV developed pressure without over loading the heart on the Frank–Starling curve.

possible to proceed into an intervention with an outcome that can be predicted to be readily reproducible.

In the case of studies investigating ischaemia/reperfusion injury, ischaemic or pharmacological preconditioning regimen may commence ahead of the induction of a period of injurious ischaemia.

Ischaemia can be induced either by complete cessation (global ischaemia) or attenuation (low-flow ischaemia) of coronary flow, or by occluding a coronary artery (typically the left anterior descending coronary artery) in regional ischaemia.

It is essential to determine the duration of the ischaemic injury in relation to the end point of the study; a short period of ischaemia may induce stunning (recoverable attenuation of myocardial function) but no infarction, while a more prolonged period of ischaemia may be so detrimental as to negate the infarct-sparing affect of any cardioprotective mechanism that may be under study.

In our hands, we find that 30–35 min of no-flow normothermic ischaemia is optimal, which in our experience results in approximately 50% infarction within the myocardium at risk, and is an infarct protocol associated with maximal reduction in infarct size following an ischaemic preconditioning stimulus. As discussed in Section 2.4.1, maintaining normothermia of the heart during this stage is crucial because it has been demonstrated that hypothermia attenuates injury [21–23].

While the presence or absence of collateral vessels between coronary vascular beds is irrelevant in the study of global ischaemia, it is highly relevant in the evolution of regional ischaemia. In guinea-pig [35–37] and hamster hearts, [37] for example the extent of collateralisation is such that ligating one of the arteries is insufficient to induce any significant ischaemia or infarction. In dog, there is up to 15–20% collateralisation, [35] but in other species, such as mouse, rat, rabbit, pig and even baboon, [36,37] the extent of collateral circulation is sufficiently limited as to enable study of regional ischaemia. Using a heart from a suitable species, experimental regional ischaemia is induced by placing a snare around the left anterior descending artery (LAD) and temporarily tightening it as seen in Fig. 3B. Achieving this reproducibly ex-vivo can be difficult in some species: in rabbit for example, the anatomy of the coronary system varies significantly from one animal to another [38] and following intravascular blood wash-out with a crystalloid buffer, the vessels are so translucent that they can be very difficult to directly visualise. One solution would be to place the snare around LAD while the heart is still in situ, where coronary vasculature is better visualized thanks to the presence of blood. Such an approach introduces additional complication,

necessitating anaesthetic and surgical procedures such as tracheal intubation, mechanical ventilation and thoracotomy. By contrast, the rat coronary system has a very constant structure [37] and the left anterior descending artery is easy to locate ex-vivo. If ischaemia is properly induced, the coronary flow and the left ventricular developed pressure should decrease by about 30% in species with a minimal collateral coronary system (such as mouse, rat, and rabbit). Consistency is required in placing the snare, so that the eventual risk zone is about 45–50% of the left ventricle volume.

Reperfusion is initiated by re-establishing coronary flow to the region at risk. In global ischaemia models, this may simply involve opening a tap or switching back on of a perfusion pump. In regional ischaemia, flow is re-established by loosening or opening the snare and may be confirmed by a (partial) recovery in flow and left ventricular developed pressure.

At the onset of reperfusion, the temperature may shift dramatically resulting in transient hypothermia or hyperthermia. Awareness of this potential complication can enable the experimenter to pre-empt the temperature shifts and avoid the potentially confounding thermal impact upon the reperfused heart.

The total duration of reperfusion is dependent upon the species under study. Myocardial necrosis is a dynamic process, with cell death not only occurring during ischaemia, but completing over a period of time during reperfusion. For the infarct to fully evolve, the duration of reperfusion needs to reflect the time-course of the pathophysiology of myocardial injury, which appears to vary from species to species. For mouse heart, many groups find 30 min of reperfusion to be sufficient. Although this period may appear inadequate for effective washout of dehydrogenases, the mouse heart has a high coronary flow rate per gram heart and studies have shown that there is no difference in infarct size following 30 or 60 min reperfusion [4]. With the rat heart where traditionally 120 min reperfusion were recommended, recent studies have shown clear infarct demarcation after 60 min reperfusion with no detectable difference in infarct size after 60 or 120 min of reperfusion [39,40].

5.1.2. Infarct size analysis

The most commonly used biological stain for infarct demarcation is triphenyltetrazolium chloride (TTC). TTC crosses the cell membranes, and binds to intracellular dehydrogenases. Viable cells with reducing potential (preserved NADPH) are coloured brick-red, while non-viable cells, with ruptured sarcolemmal membranes and enzyme wash out, appear pale. Thus TTC-treated heart slices are ideal for macroscopic infarct size assessment.

5.1.2.1. Regional ischaemia staining protocol.
Following regional ischaemia, a double staining strategy is necessary. Firstly, at the end of reperfusion, the suture around LAD is tightened and Evans blue (0.25% in saline) is infused via the cannula into the coronary system. The dye will colour the non-risk zone dark blue, while the risk zone (in which the dye does not have access) remains pale pink (Fig. 3C). Thereafter, the TTC staining protocol (as described in the following section) may be applied, staining viable myocardium brick red, non-viable and unstained myocardium appearing pale within the risk zone (Fig. 3D).

5.1.2.2. Global ischaemia staining protocol.
Following global ischaemia, single staining with TTC alone is used to differentiate necrotic versus viable myocardium. There are two approaches taken here. For larger hearts, such as rat and rabbit, the hearts are decannulated at the end of the experimental protocol and frozen at −20 °C. The hearts are then sectioned with a slice thickness of 2 mm, perpendicular to the long axis, and incubated in a warmed, 37 °C, 1% TTC in phosphate buffer (pH 7.4) for 10 min. For smaller hearts such as those from mouse, where the slice thickness is necessarily thinner (1 mm), the heart is first infused with TTC via the aortic cannula, before being placed in 1%

TTC solution at 37 °C for 10 min. The difference in protocol compared to large heart preparations being that we found that thinner heart slices tended to curl on themselves during incubation making subsequent analysis difficult. The mouse hearts are then frozen and stored at −20 °C. Within 24 h the hearts are sliced transversally into 1 mm slices.

An alternative approach for small hearts is to fix the heart in wax and use a microtome to slice the fixed hearts. Pre-incubation in TTC as described above is nevertheless necessary.

The next stage is to remove excess TTC staining from non-viable tissue, which has the benefit of increasing the contrast between viable and non-viable tissue. This is achieved by placing the stained myocardium overnight in formalin (40% formaldehyde in 0.9% NaCl) for a clearer delineation between the viable (red) and nonviable (pale) tissue. It is recommended that the heart slices should not be kept in formalin longer than 24 h before infarct size computation to avoid the discolouration which will affect the infarct size measurement.

5.1.3. Infarct size measurement

The heart slices are arranged from apex to base and compressed between two transparent plates spaced apart at a pre-determined distance (1–2 mm), then digitally photographed or scanned on a flat-bed scanner. Once the image is calibrated with a graduated scale, it enables slice areas to be converted into slice volumes. The digital heart slice images may then be imported into a proprietary graphics program (for example, NIH free share image analysis program, NIH Image http://www.rsb.info.nih.gov/nih-image) to enable the planimetry of the heart slices. The volumes of viable and non-viable tissue within the region at risk can be ascertained, and the final result is expressed as an infarct to risk zone ratio (I/R%).

5.2. Western blot analysis

Langendorff perfusion (in either constant pressure or constant flow mode) is an ideal way of removing unwanted blood cells and protein from the explanted heart prior to tissue freezing. Study procotols should be designed in cognisance of the potential stress activation and phosphorylation that occurs during the process of harvesting and hanging the isolated heart upon the Langendorff rig [33,34]. A time-course characterisation of the stress activation profile following Langendorff perfusion of the protein under study is therefore recommended, which will aid in the further interpretation of the data. For Western blot of proteins following prolonged ischaemia/reperfusion protocols, transient activation of stress proteins appears to be less of a problem. In all cases, the hearts can be rapidly snap frozen with liquid nitrogen with minimal delay from perfusion.

5.3. Myocyte isolation

The Langendorff perfusion technique provides a methodology for harvesting viable cardiac myocytes from whole hearts. The apparatus is essentially the same as used for other perfusion studies, and is run in the constant flow mode—an important consideration, as digestion with collagenases will impair normal vascular function. As with other manifestations of the technique, rapid and efficient cannulation and transfer of the heart to the Langendorff apparatus is essential for a good quality myocyte preparation.

There are clear differences in the buffers employed in myocyte isolation, with sequential perfusion in two or three different buffers. In our laboratory, we tend to proceed directly to calcium-free perfusion, but many others perfuse first in a calcium containing buffer to permit contraction of the heart to expel blood, before proceeding to calcium free perfusion. Our calcium free buffer contains (in mmol/l) NaCl 113.0, KCl 4.7, $KH_2PO_40.6$, $Na_2HPO_40.6$, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 12.0, $KHCO_3$ 10.0, taurine 30.0, HEPES 10.0, and glucose 5.5. The digestion step is performed with a collagenase perfusion

buffer containing collagenase type II 0.9 mg/ml, hyaluronidase 0.125 mg/ml, and $CaCl_2$ 12.5 μmol/l. Suspended hearts are thus Langendorff perfused sequentially with oxygenated (95% O_2/5% CO_2), warmed buffer (37 °C). In our hands, we use 4 and 10 min for calcium-free and digestion buffers respectively.

By the end of the second perfusion period, the heart displays morphological distortion, at which stage the heart is decannulated, the ventricles cut into several pieces and the sectioned heart shaken in oxygenated collagenase buffer for a further 10 min at 37 °C. Thereafter, the supernatant is collected, 5% foetal calf serum added, centrifuged at 600 rpm for 3 min and resuspended before the calcium concentration gradually restored (to avoid the calcium paradox) to 1 mmol/l over a period of 20 min.

Thus prepared, Langendorff-perfusion primed isolated myocytes can then be plated for subsequent study using a variety of methodologies, from microscopy techniques, such as confocal, to further in-vitro investigations such as simulated ischaemia/reperfusion (review [41]), where removal from the extracellular matrix is deemed desirable, or gene-transfer/gene-silencing using viral or non-viral vectors required (review [42]).

5.4. Measurement of electrical activity

The Langendorff perfused heart offers an excellent opportunity to simultaneously monitor function and electrical activity, largely thanks to the excellent access to the epicardium that the preparation provides. This enables thorough characterisation of the regional variation of the electrical properties/ action potentials of the whole heart [43]. Moreover, application of a drug or altering the constitution of the perfusion buffer, enables study of the electromechanical association, comparing contractile function with, for example, alterations of electrical conduction through the AV node and Purkinje fibres (PR interval and QRS duration) [44]. Langendorff perfused hearts, with ECG monitoring are also used, through monitoring of the QT interval, in drug screening systems such as SCREENIT™ for potential arrhythmogenesis of new drug compounds (review [45]). Such applications can prove to be a very powerful tool not only of normal physiology, but also in the examination of pathological states such as heart failure, and the subsequent understanding and characterization of the evolution of arrhythmia (review [46]).

With the appropriate choice of animal species, mindful of potential clinical relevance [13], it is possible to map electrical activity across the heart using simple electrodes to monitor monophasic action potentials or record surface electrocardiograms to further develop understanding of the initiation, maintenance and termination of serious arrhythmias [46]. Moreover, it is possible, with the use of potentiometric dyes or microelectrode arrays (which can be applied to hearts of various species, from mouse [47] and rat [48] to human [6]), to map electrical wave fronts and determine conduction velocities in the evolution of ventricular or atrial arrhythmia.

6. Conclusions

From its introduction at the end of the 19th century to the present day, the Langendorff perfused heart model has proved to be an irreplaceable tool for research into contractile and electrical/conduction properties and coronary vascular function in physiological, pathological or pharmacological investigations, and into myocardium biochemistry and metabolism. Flexible and adaptable, the system has also provided a useful tool for isolating different cell populations from the heart or for the optimum preparation of the myocardium for electron microscopy, and, last but not least, the investigation of cardioprotective interventions against ischaemia–reperfusion injury.

Langendorff perfusion has a number of disadvantages which the adept investigator can turn to an advantage in the study of particular parameters: the isolated perfused mammalian heart nonetheless provides an essential bridge between in-vitro investigations and in-vivo experiments. The valuable information gathered by using this technique, when subsequently validated by in-vivo animal models, enable researchers to make further steps towards the ultimate goal of translating knowledge of the basic science to human benefit.

Disclosures

None.

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