A Closed Circulation Langendorff Heart Perfusion Method for Cardiac Drug Screening

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Summary
Cardiovascular diseases represent an economic burden for health systems accounting for substantial morbidity and mortality worldwide. Despite timely and costly efforts in drug development, the cardiovascular safety and efficacy of the drugs are not always fully achieved. These lead to the drugs' withdrawal with adverse cardiac effects from the market or in the late stages of drug development. There is a growing need for a cost-effective drug screening assay to rapidly detect potential acute drug cardiotoxicity. The Langendorff isolated heart perfusion technique, which provides cardiac hemodynamic parameters (*e.g.,* contractile function and heart rate), has become a powerful approach in the early drug discovery phase to overcome drawbacks in the drug candidate's identification. However, traditional *ex vivo* retrograde heart perfusion methods consume a large volume of perfusate, which increases the cost and limits compound screening. An elegant and cost-effective alternative mode for *ex vivo* retrograde heart perfusion is the constant-flow with a recirculating circuit (CFCC), which allows assessment of cardiac function using a reduced perfusion volume while limiting adverse effects on the heart. Here, we provide evidence for cardiac parameters stability over time in this mode. Next, we demonstrate that our recycled *ex vivo* perfusion system and the traditional open one yield similar outputs on cardiac function under basal conditions and upon β-adrenergic stimulation with isoproterenol. Subsequently, we validate the proof of concept of therapeutic agent screening using this efficient method. β-blocker (*i.e.,* propranolol) infusion in closed circulation countered the positive effects induced by isoproterenol stimulation on cardiac function.

Keywords
Drug development • Drug screening • Cardiovascular safety • Langendorff method • Closed circulation

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Introduction

During the last century, adverse effects associated with many drugs in clinical use have arisen as a major safety concern in the pharmaceutical industry, resulting in the withdrawal of several previously approved drugs from the market [1]. It has been reported that adverse drug effects are dependent on the sex, age, and genetic background of the individual [2]. There is an urgent need to develop new, tailored, and safe therapies. Ideally, potential adverse effects should be tested on isolated organs. Drug development is a long and expensive process during which, one of the main challenges remains the prediction of drug-induced cardiotoxicity [3]. The discovery of a cardiotoxic effect is sufficient to halt drug development and the clinical management of existing drugs [4]. Manifestations of cardiotoxicity response include cardiac arrhythmias, hypertrophy, and may even progress to heart failure (HF). Drug screening methods using animal or cellular models have historically been implemented in drug approval procedures. However, these preclinical studies may be a source of limitations in accurately predicting drug performance in human patients and may mask some cardiovascular safety concerns [5]. Following the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, the evaluation of hemodynamic effects and pro-

arrhythmic potential of all non-cardiac drug candidates is mandatory before approval [6]. It has been reported that therapeutic agents with cardiotoxicity usually affect cardiac contractility, mitochondrial function, or cellular signaling [7]. This emphasizes the importance of investing in cost-effective tests to screen effective drug candidates and their cardiovascular safety in the early stages of development.

Studies on isolated hearts (*e.g.*, Langendorff and working heart preparations) should be considered the ideal bridge between *in vitro* and *in vivo* experiments, offering applications in cardiac physiology and pharmacology [8,9]. Langendorff perfusion studies, originally described by Oscar Langendorff in 1895, have been a cornerstone of cardiovascular research [10]. Since the end of the 19th century, Langendorff perfusion has been a versatile tool for in-depth study of the heart, from basic physiology to pathological cardiac conditions, but also an asset for deciphering the pharmacological effects of drugs on cardiac function [11,12]. In contrast to the working heart model, the Langendorff method relies on retrograde circulation of the perfusion buffer in the aorta to supply the myocardium, via the coronary vascular bed, with oxygen and nutrients needed to maintain cardiac function. *Ex vivo* methods for retrograde perfusion of hearts include mainly constant pressure and constant-flow configurations [11]. In constant pressure mode, the perfusion pressure is maintained by elevating the buffer reservoir (~ 1 m) to reach 60 mmHg. While in the constant-flow mode, a peristaltic pump ensures regular perfusate flow through the coronary vasculature at a speed in ml/min of 7.43*(Heart Weight)$^{0.56}$ [13]. Therefore, changes in coronary resistance are identified as blood flow modifications in the first mode and as pressure changes in the second. Thus, the latter is preferred for coronary vascular tone studies. In both systems, changes in left ventricular pressure during diastole and systole can be monitored after the insertion of a fluid-filled balloon catheter into the left ventricle, which measures cardiac parameters including the developed left ventricular pressure (dLVP), the left ventricular end-diastolic pressure (LVEDP), the maximal contraction velocity (dP/dt_{max}), the maximal relaxation velocity (dP/dt_{min}), the heart rate (HR), and rate pressure product (RPP). In addition, electrical activity and conduction information can be investigated by optical mapping [14]. Early assessment of electrophysiologic and hemodynamic effects of drug candidates at the drug discovery phase prevents drug withdrawal from the late stage of clinical trials or even from the market. However, traditional *ex vivo* methods (*i.e.,* constant pressure and flow) to record cardiac parameters require large perfusion volumes, which further hinders access to screening for expensive molecules.

We present a detailed retrograde heart perfusion protocol using constant-flow and closed circulation (CFCC) for consistent drug screening and reliable data analysis. Our protocol includes specific steps for initiating CFCC heart perfusion, as well as procedures for recording and analyzing cardiac parameters. The use of a recycling perfusion system reduces the cost of drug screening without affecting cardiac parameters. Therefore, a wide range of pharmacological agents (e.g., sympathomimetic, parasympathomimetic, coronary vasodilator, and cardiac glycol-sides) can be tested alone or in combination to investigate the pharmacological effects on the heart [15]. To our knowledge, no studies are testing the reliability of the *ex vivo* retrograde cardiac perfusion method in CFCC for drug screening. We provide evidence supporting the reliability of the CFCC heart perfusion method in terms of stability of cardiac parameters and as a tool of choice for screening pharmacological agents to identify acute toxic effects. This method can be extended to other species of mammals, including humans using explanted hearts following transplantation, making it possible to assess the cardiac response to various therapeutics or molecules in isolated hearts, without the influence of other organ systems [16]. Each class of therapeutic drugs can have unforeseen cardiotoxicity, thus the application of this type of method should become a standard procedure as a prerequisite in drug discovery research.

Methods

Animals

Adult male Wistar rats (6-8-week-old, Janvier Labs, Le Genest-Saint-Isle, France) were housed in a temperature-controlled room and exposed to a 12 h/12 h light/dark cycle with *ad libitum* food and water consumption. All animal care and procedures were conducted in agreement with European guidelines for animal welfare with the approval of the French Ministry of Agriculture and Food and Local Ethics Committee (agreement n ° A 91-471-109).

Perfusion system preparation

As prerequisite to *ex vivo* heart perfusion assay, an air bubble trap and a left ventricular pressure balloon were made, solutions (*i.e.*, Krebs-Henseleit buffer (KHB),

cannulation buffer, and pharmacological agent preparation) were prepared. The setup calibration needs to be performed before proceeding to *ex vivo* assays. A list of needed materials and equipment is available in Supplementary Table 1. In addition, a detailed description of each step and buffer is listed in Supplementary Methods and Supplementary Table 2.

The perfusion system was put into operation, air bubbles were removed and the air bubble trap was placed (Fig. 1A). The peristaltic pump providing the recirculation circuit was adjusted to deliver the perfusate at a flow rate 1.5 to 2 times higher than the physiological flow rate (*i.e.*, 10 ml/min). The coronary flow is calculated using the formula: flow (ml/min) = 7.43* Heart weight$^{0.56}$, Heart weight = 0.0027* Body weight + 0.6 [17]. Reservoirs were filled with warm oxygenated KHB (Fig. 1A). The peristaltic pump maintains the KHB supply in the reservoir (Fig. 1A).

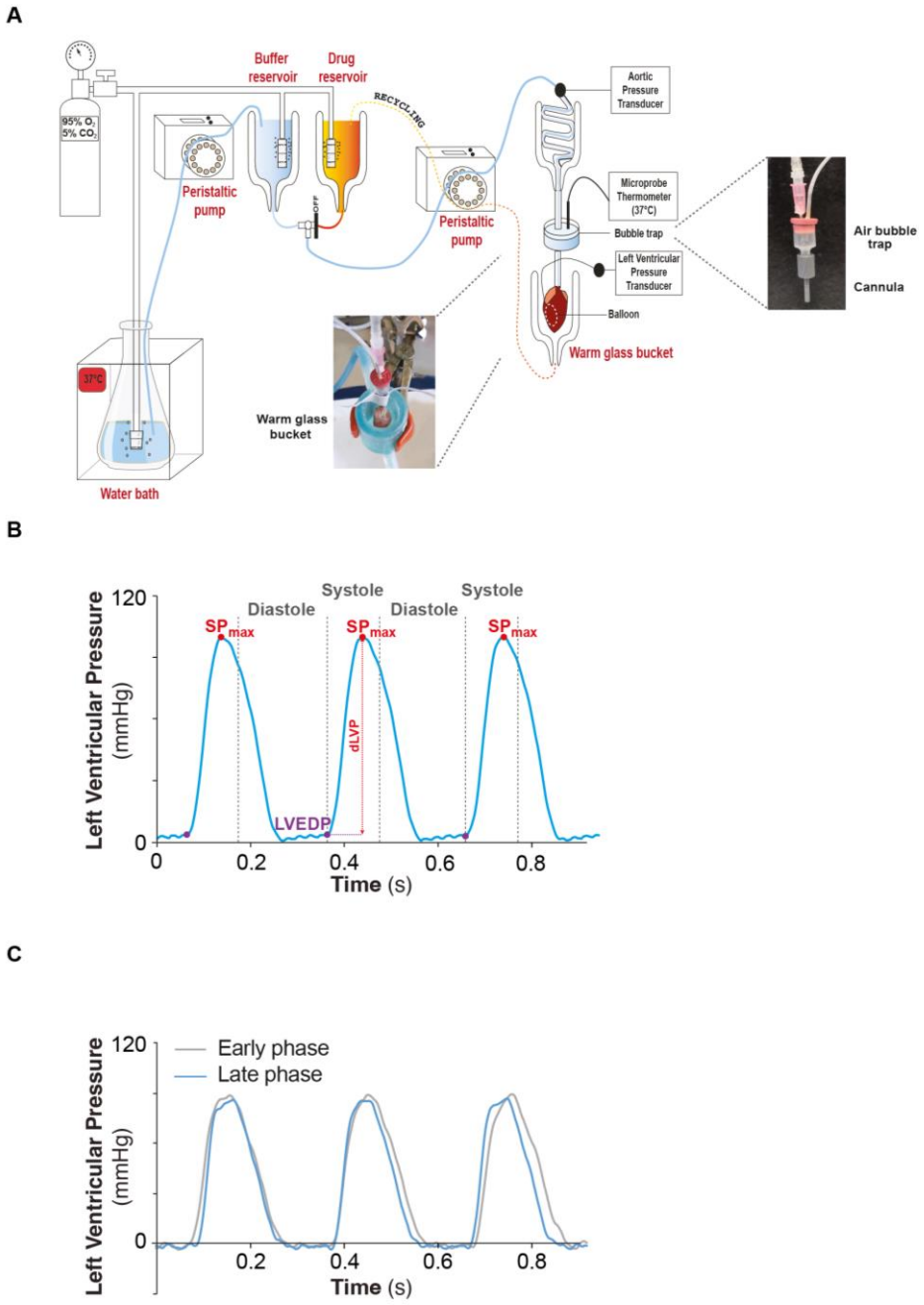

Fig. 1. *Ex vivo* retrograde-perfused heart setup in constant-flow and closed circulation (CFCC).
(A) Scheme of the retrograde heart perfusion system in CFCC; **(B)** Left ventricular pressure trace showing the left ventricular end diastole pressure (LVEDP; purple circle), the developed left ventricular pressure (dLVP; dashed red line) and the maximum systolic pressure (SP_{max}; red circle); **(C)** Representative traces of left ventricular pressure were recorded post-cardiac stabilization (early phase; light grey) and 1h later (late phase; light blue).

Anesthesia, excision of the heart, and cannulation of the aorta

Rats of 300 g were anticoagulated, anesthetized by an intraperitoneal injection of Euthasol (80 mg/kg of pentobarbital sodium), and placed in the supine position. The heart was removed quickly and the ascending aorta was mounted on the cannula, secured by ligating twice with a double surgical silk ligature (Supplementary Fig. 1A). Then, the aortic cannula with the heart was carefully mounted on the Langendorff apparatus in operation and dispersing oxygenated KHB solution from the reservoir. The apex of the heart was pierced with a needle to avoid overpressure. Hearts were suspended from their aortic cannula and maintained warm at 37 °C in a hot glass bucket (Fig. 1A and Supplementary Methods).

Retrograde heart perfusion and acquisition

Hearts were retrogradely perfused through the aorta with oxygenated KHB and at a constant-flow (Supplementary methods). During the perfusion, IOX software v1.8 (EMKA Technologies, Paris, France) provided data acquisition, analysis, and storage from the perfusion system. A catheter connected to a pressure transducer was placed upstream of the aorta to record the coronary perfusion pressure (CPP). The basal CPP (~80 mmHg) was recorded throughout the experiments after the heart stabilization (~10 min). This recording monitors the adaptability of coronary pressure in hearts exposed to various substances. The filled left ventricular pressure balloon was inserted into the left ventricle and connected to a pressure transducer, which mediated the dLVP (mmHg) recording. For the filled left ventricular pressure balloons, non-elastic materials made of ultra-thin plastic film (e.g., commercial wrapping foil), usually homemade, are recommended as they give a higher value of contractile parameters than commercial latex balloons [18,19]. However, these handmade balloons present significant inter- and intra-individual manufacture variability (e.g., shape, volume, leakage), and their handling requires considerable expertise. In contrast, commercial latex balloons are ready-to-use and guarantee better reproducibility of recordings through standardization of manufacture. dLVP was calculated from the maximum systolic pressure (SP_{max}) minus LVEDP. The size of the pressure balloon was increased until the dLVP plateaued, indicating that the balloon fit completely into the left ventricular cavity. Notably, the LVEDP is a marker of left ventricular dysfunction in hemodynamic assessment [20]. We have therefore named the early phase, which follows heart stabilization, and the late phase which corresponds to 60 min after stabilization. In general, the data referring to basal were collected 60 min after stabilization and were followed by stimulation steps (*e.g.*, β-adrenergic agonist). IOX software provided maximum positive (dP/dt_{max}) and negative (dP/dt_{min}) values of the first derivative of left ventricular pressure that occurs during the cardiac cycle (in mmHg/sec), the heart rate (HR, bpm) and the rate pressure product (RPP), which is an index of cardiac mechanical performance (in mmHg*bpm). RPP = (dLVP x HR)/1000 [21]. Exclusion criteria for the perfused rat heart assays included cannulation time >3 min, and after cannulation in the basal state, presence of continuous arrhythmias >5 s, or an RPP <10 or >25 mmHg*bpm [11,22].

Pharmacological applications

After heart stabilization and basal recordings, drugs of interest were administered throughout the circulating perfusion solution to assess the effects of these drugs on cardiac functional parameters. Drugs were diluted in the oxygenated KHB at the desired concentration (*i.e.*, propranolol: 1 µM (Merck-Millipore, France) [23]). The drug solution was spilled into a second reservoir (Fig. 1A). Then, the first perfusion circuit was switched to the one containing the drug of interest. Hearts were perfused at a constant-flow rate and in a closed circuit for 1 h during which cardiac functional parameters were recorded. The recirculation time should be adapted for each drug. Additional drug infusion can be performed simultaneously or in a staggered fashion to study synergistic or combined effects. β-adrenergic stimulation of cardiac muscle was performed with isoproterenol (Iso, Merk-Millipore, France) administrated at 10 nM for 3 min. Data were collected at the end of the stimulation stage.

End of acquisition

Hearts were removed from the cannula (see Supplementary Methods for detailed steps), and could be rapidly frozen for further analyses (*e.g.*, immunoblot, immunofluorescence, RT-qPCR). The setup was thoroughly washed.

Data analysis

Values of cardiac functional parameters (dLVP, LVEDP, dP/dt_{max}, dP/dt_{min}, HR, and RPP) were extracted by IOX software and proceeded for statistical analyses with GraphPad Prism 9 (v9.1.0). Statistical tests were performed using two-way ANOVA with a Bonferroni posthoc test. Statistical significance was assumed with $p<0.05$.

Results

Ex vivo perfused heart model in CFCC

Langendorff retrograde perfusion system is a refined and well-known method for recording cardiac parameters in isolated hearts without the influence of other organs. Traditionally, Langendorff in constant pressure and constant-flow relies on the use of a large volume of perfusion, which may be limiting for drug screening or testing expensive molecules. An alternative to overcome this limitation is heart perfusion in a constant-flow incorporating a recirculating closed circulation (CFCC). This lesser-known system reduces the perfusion volume while increasing the heart exposition to the molecules of interest. However, to the best of our knowledge this system suffers from a lack of comparative studies between *ex vivo* systems on cardiac functional parameters that would ensure the absence of cardiac distress. A schematic representation of the *ex vivo* heart system in CFCC that we used in this study is shown in Fig. 1A. A detailed protocol for this procedure is provided in the Supplement Methods. Fig. 1B shows a left ventricular pressure trace recorded with a ventricular balloon during an *ex vivo* heart perfusion procedure in CFCC, which provides cardiac functional parameters, including dLVP, LVEDP, HR and RPP. dLVP corresponds to the difference between the maximum systolic pressure (SP_{max}) and LVEDP. The maximal contraction and relaxation velocities (dP/dt_{max} and dP/dt_{min}, respectively) are calculated as the maximum values of the left ventricular pressure first derivative during contraction and relaxation, respectively.

Cardiac functional parameters are stable in the Ex vivo heart model perfused in CFCC

Cardiac functional parameters collected with traditional *ex vivo* retrograde heart perfusion methods are known to remain stable over time (~ 1h) [24]. Therefore, we tested the stability of these parameters in the CFCC cardiac perfusion system at baseline. Isolated rat hearts were perfused in CFCC perfusion mode, during which LVP was recorded. Next, cardiac functional parameters corresponding to the time post-cardiac stabilization (early phase) and after an hour of recirculation (late phase) were analyzed (Fig. 1C and Table 1). We observed an LVEDP that ranged between 0-10 mmHg indicating the absence of heart damage and reported an overlap of the LVP traces with a stable HR between the early and late phase of perfusate recirculation (Fig. 1C and Table 1). Cardiac functional parameters remained stable over 60 min period (Table 1) but started to drop after ~90 min with up to 50 % reduction in LVP after 120 min of perfusion (not shown). Thus, for safety, all experiments with the CFCC perfusion mode were limited to 60 min duration.

Comparison of cardiac functional parameters in CFCC mode and in constant-flow with open circulation (CFOC)

To further characterize the *ex vivo* retrograde-perfused cardiac system in CFCC, we compared the cardiac functional parameters collected at basal and after β-adrenergic stimulation with 10 nM isoproterenol (Iso) in this mode and in the constant-flow with open circulation mode (CFOC) (Fig. 2). The time course of left ventricular pressure traces recorded in CFCC and CFOC modes (Fig. 2A blue and grey traces respectively) showed a similar progressive increase in response to Iso infusion with clear positive chronotropic, inotropic, and lusitropic effects.

One hour after heart perfusion, we observed no differences in cardiac contractility (HR, dLVP, dP/dt_{max}, dP/dt_{min}, and RPP) either at basal or after Iso stimulation between CFCC and CFOC modes (Fig. 2B-F, blue and grey bars respectively). Therefore, Iso induced a similar and significant increase ($p<0.001$) of

Table 1. Cardiac hemodynamic parameters remain stable over time in the *Ex vivo* heart model perfused in constant-flow and closed circuit (CFCC). Rat hearts were mounted in CFCC mode and cardiac hemodynamic parameters (dLVP, dP/dtmax, dP/dtmin, HR and RPP) were recorded after heart stabilization (early phase) and after 60 min recycling (late phase). Data show the mean ± SD (n=3 independent experiments; ns for non-significant).

		dLVP (mmHg)	**dP/dt_{max}** (mmHg/s)	**dP/dt_{min}** (mmHg/s)	**HR** (bpm)	**RPP** (mmHg*bpm)
Early phase	Mean ± SD	74.6 ± 7.6	2417.5 ± 321.4	-1239.7 ± 115.5	220.4 ± 22.5	15.9 ± 2
Late phase	Mean ± SD	74.2 ± 6.4	2637.7 ± 301.4	-1265,4 ± 83.5	219,4 ± 35.8	15.9 ± 1.6
p value		0.95 ; ns	0.43 ; ns	0.77 ; ns	0.96 ; ns	> 0.99 ; ns

cardiac contractility *vs.* basal in both configurations: HR was increased by ~30 %, dLVP by >55 %, dP/dt_{max} by >80 %, dP/dt_{min} by >110 %, and RPP by >100 % (Fig. 2B-F). These results indicate that the cardiac perfusion system in CFCC mode is as efficient as the traditional CFOC for collecting cardiac functional parameters. As expected, Iso perfusion reduced coronary pressure (Supplementary Fig. 1F). As such, its use appears to be an asset for screening therapeutic agents.

Propranolol attenuated isoproterenol effects on cardiac functional parameters in CFCC mode

Next, we tested the proof-of-concept that recirculation of molecules in CFCC-perfused hearts is ideal for testing their impact on cardiac function and could thus serve as a powerful tool for drug screening. Therefore, we measured the effects of one-hour recirculation of a non-selective β-blocker (1 µM propranolol) on cardiac function (Fig. 3). This choice is supported by the well-documented effects of propranolol on cardiac functional parameters. As previously observed in Fig. 2, the time course of recorded left ventricular pressure traces (Fig. 3A) showed a progressive increase in response to Iso infusion in the vehicle condition (Fig. 3A, blue traces). As expected, this increase was blunted in the propranolol recirculation condition (Fig. 3A, green traces). We observed similar effects on cardiac function (HR, dLVP, dP/dt_{max}, dP/dt_{min} and RPP) at baseline between vehicle and propranolol recirculation (Fig. 3B-F, green and blue bars respectively).

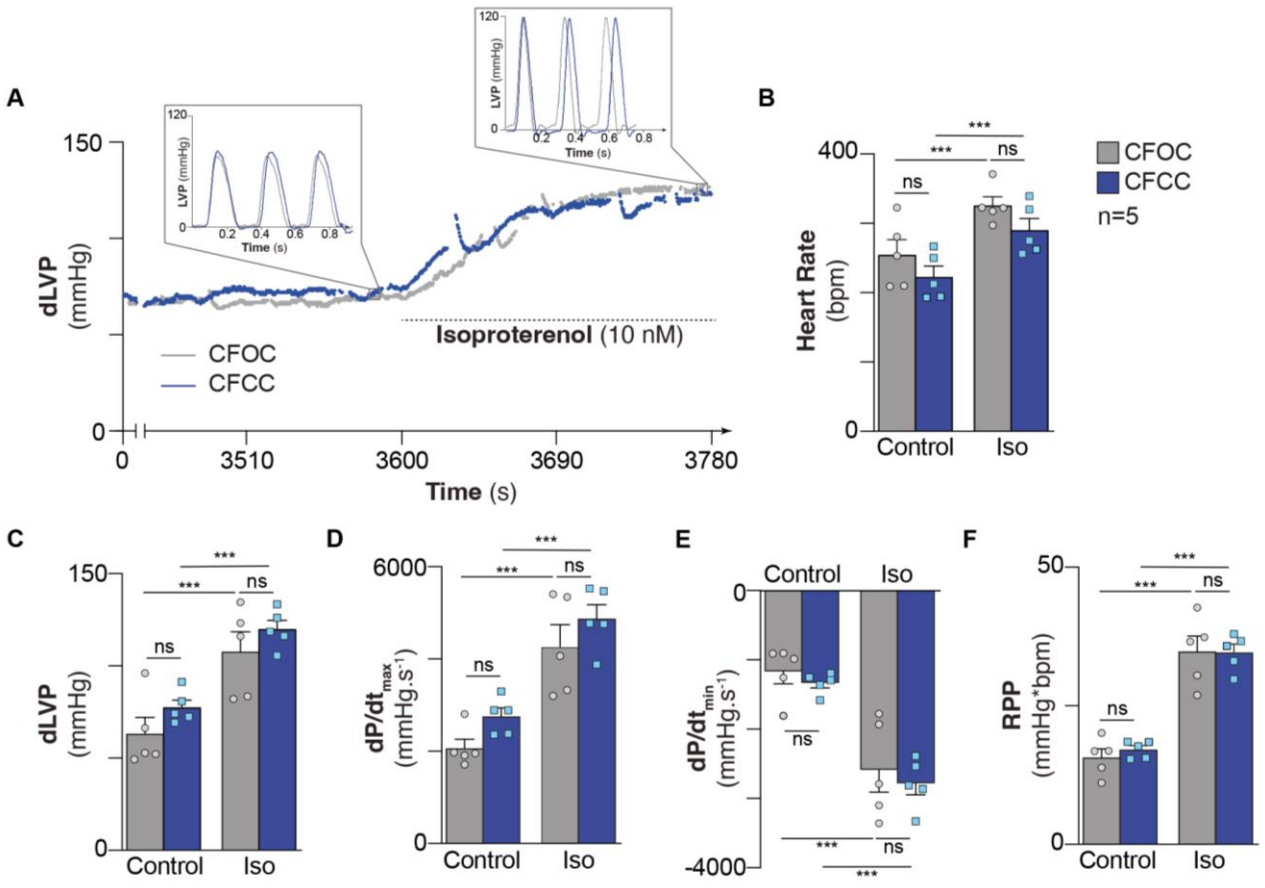

Fig. 2. Perfused rat heart in constant-flow and closed circulation (CFCC) mode shows similar cardiac functional parameters compared to constant-flow and open circulation (CFOC) with or without β-adrenergic stimulation.
(**A**) Time course traces with zoomed sections of left ventricular pressure from perfused rat hearts in closed (dark blue) or open (grey) circulation with or without isoproterenol stimulation (10 nM). (**B-F**) Amalgamated data extracted from traces showing the heart rate (**B**), the developed left ventricular pressure (dLVP) (**C**), the maximal contraction velocity as dP/dt_{max} (**D**), the maximal relaxation velocity as dP/dt_{min} (**E**), and the rate pressure product (RPP) (**F**). Data show the mean ± SEM (n=5 independent experiments; ns for non-significant, ** p<0.01).

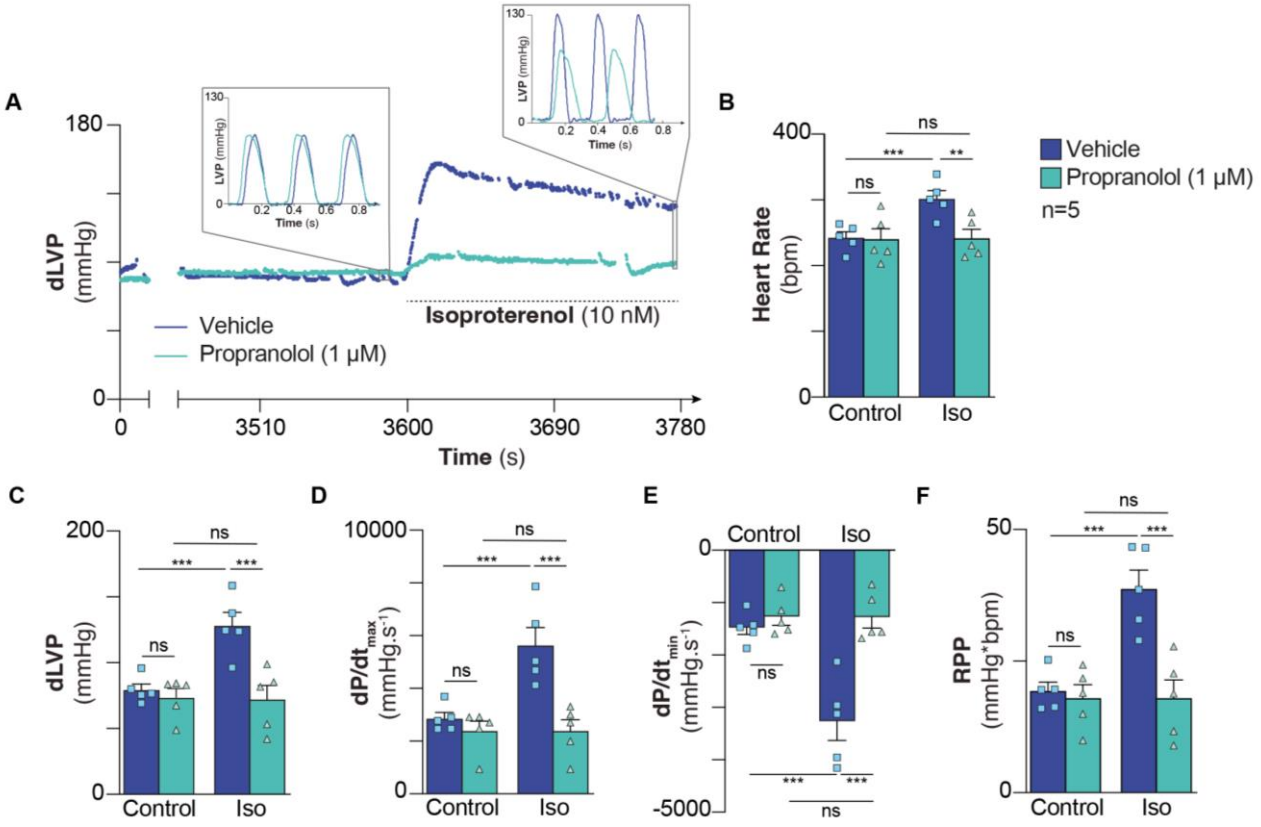

Fig. 3. Recirculated propranolol reduces cardiac functional parameters of perfused rat hearts in closed circulation under β-adrenergic stimulation.
(**A**) Time course traces with zoomed sections of left ventricular pressure from perfused rat hearts in closed circulation in the presence (green) or absence (dark blue) of propranolol (1 µM) with or without isoproterenol stimulation (10 nM). (**B-F**) Amalgamated data extracted from traces showing heart rate (**B**), developed left ventricular pressure (dLVP) (**C**), maximal velocity of contraction (dP/dt_{max}) (**D**), and relaxation ($-dP/dt_{min}$) (**E**), and rate pressure product (RPP) (**F**). Data show the mean ± SEM (n=5 independent experiments; ns for non-significant, * p<0.1, ** p<0.01 and *** p<0.001).

In comparison to basal, isoproterenol infusion significantly increased HR (~25 %; p<0.001) and dLVP (>60 %; p<0.001), accelerated the kinetics of contraction (~97 %; p<0.001) and relaxation (~120 %; p<0.001), and increased RPP (~100 %; p<0.001) in the control group (Fig. 3A, blue trace, and Fig. 3B-F, blue bars). However and as expected, these positive effects of isoproterenol on cardiac parameters were completely blocked in the group re-perfused with propranolol compared with vehicle (-20 % in HR, p<0.01; -43 % in dLVP; p<0.001; -57 % of dP/dt_{max}; p<0.001; -54 % in RPP and an increase by 60 % in dP/dt_{min}; p<0.001) (Fig. 3B-F). In addition to these, we showed no differences in cardiac parameters collected in the propranolol re-perfused group with or without isoproterenol stimulation (Fig. 3, green trace in A, and green bars in B-F). Therefore, our data supports the *ex vivo* retrograde perfused heart system in CFCC as a powerful method for screening new therapeutics and assessing their effects on cardiac function.

Discussion

The Langendorff method has led to numerous important advancements in cardiac physiology, including the understanding of heart contractile function, regulation of coronary blood flow, and cardiac metabolism [11]. This *ex vivo* isolated perfused heart method has been used to investigate cardiac pathological models (*e.g.*, ischemia/reperfusion) and for pharmacological studies [25,26]. In recent times, advances in molecular biology and genetic manipulation in combination with Langendorff method have led to deciphering the complexity of mechanisms underlying human cardiac diseases [19]. Although informative with their specificities, two-dimensional (2D) monolayer cell cultures of primary cardiomyocytes or cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CM) do not fully reproduce the three-dimensional (3D) cardiac microenvironment [27]. Thus, 3D cardiac models such as human-engineered heart tissue, which

constitute an emerging field, could solve some issues but they still require optimization [28-30]. Therefore, the *ex vivo* Langendorff perfusion methods bridge the gap between *in vitro* monolayer cultures and *in vivo* animal model studies. Among the *ex vivo* heart perfusion methods, constant-flow and pressure configurations are commonly used. They enable cardiac function to be studied without the influence of organs including signals from the central and autonomic nervous systems, which results, in the absence of external stimulation (*e.g.*, drugs or electrical stimulation), in HR reduction compared with that reported *in vivo*. Traditionally, *ex vivo* methods operate in open perfuse circuits, which can be limiting for some specific studies including the effects of compounds on cardiac function or drug screening. A pertinent adaptation is to recycle perfusate in a closed circuit. However, additional comparative studies are required to evaluate the different *ex vivo* methods, especially regarding the stability of cardiac functional parameters in comparison to traditional systems. We have provided evidence that the retrograde-perfused system in CFCC is a reliable method that overcomes the limitations of CFOC systems. It is therefore an asset for drug screening and underpinning their acute effects on cardiac function. In our conditions, we showed CFOC and CFCC exhibit similar cardiac functional parameters at basal and after β-adrenergic stimulation. We observed that Iso induced significant inotropic, chronotropic, and lusitropic effects in perfused hearts, as anticipated. In CFOC and CFCC systems the recorded dLVP and LVEDP ranged within expectable values (70-130 and 0-10 mmHg, respectively) indicating adequate myocardial contractile force [11]. A high LVEDP value of greater than 10 mmHg indicates cardiac damage. In addition, the RPP, commonly used as an indicator of heart function, was found to be similar between CFOC and CFCC. As expected, Iso increased RPP values, illustrating a higher metabolic demand. However, in case of constant-flow perfusion, there is no simultaneous increase in coronary flow despite a decrease in coronary pressure. This limitation may restrict the inotropic response due to inadequate oxygen supply [11,19]. Besides exhibiting comparable cardiac functional parameters, the CFCC *ex vivo* model has the added advantage of using a low volume of recycled perfusate. This system is ideal for conducting *ex vivo* cardiac pharmacology studies, including the identification of new drug candidates and their evaluation on cardiac function. This includes assessing anti-arrhythmic versus pro-arrhythmogenic effects, cardioprotective versus cardiotoxic effects, and contractility worsening. This is supported by the recycling of propranolol, which blunted the effect of Iso on cardiac functional parameters [31]. On the other hand, the absence of propranolol effects at basal was expected, showing that the heart is free from the influence of signals from the central and autonomic nervous systems. Furthermore, all traces of neurotransmitters (specifically noradrenaline and acetylcholine) were eliminated during heart stabilization [16]. We have shown the CFCC system to be effective for drug screening strategies, but it must be coupled with *in vivo* pharmacokinetics and pharmacodynamics assays.

One limitation of the CFCC model is the release and accumulation, over time, of metabolites from the heart in the recirculating buffer. This could bias the recorded cardiac parameter values throughout the experiments. One way to monitor this is to sample the recirculation medium over time and quantify various factors including troponin T, nucleotides (ATP, ADP, AMP), creatine phosphokinase, myoglobin, NT pro-BNP [32,33]. Increased release of these compounds may reflect cardiac distress and have possible deleterious consequences on cardiac functional parameters [33-35]. Previous studies on isolated hearts perfused under CFCC conditions have shown that the accumulation of some of these metabolites in the recycling medium increases significantly after 90 min of reperfusion (LDH, Troponin, myoglobin, ammonia) [35,36]. These results align with our observations, where in our CFCC perfusion conditions cardiac parameters are stable over 90 min of recirculation, after which we observe a decrease in cardiac parameters. Of note, contractile and chronotropic functions decline by 5-10 % per hour in open circulation systems [9]. Countermeasures include adaptation of perfusion volume and perfusate filtration. Additional factors that may affect cardiac functional parameters during *ex vivo* experiments, such as maintaining heart temperature and preventing air bubbles in the perfusate, need to be considered.

Outside the thoracic cavity, the heart undergoes radiant heat loss leading to hypothermia, affecting myocardial contractility and heart rate [37]. Therefore, the perfusion solution flowing through the coronary vascular bed must be pre-warmed and the cannulated heart must be exposed to an external heat source (*e.g.*, with a heated glass bucket). The presence of air bubbles in the coronary vasculature triggers air embolism, which can be prevented by a bubble trap upstream of the cannulated heart [11]. Moreover, the time between heart excision and perfusion needs to be minimized (<3 min) to avoid ischemic preconditioning of the heart [38]. Finally, poorly

controlled excision surgery or inadequate preparation of the heart can lead to heart failure, which can be easily detected on traces showing sudden changes in cardiac parameters [37].

The protocol described here applies to healthy and pathological hearts (*e.g.*, myocardial infarction, ischemia-reperfusion, HF) and can be customized for all animal species, from mice to dogs and pigs, and even human hearts [39]. Additional considerations specific to each species must be taken into account, such as coronary flow rate, total perfusion duration, cannula size, and perfusate volume, which vary across different species [40].

These data suggest that the closed-circuit, constant-flow *ex vivo* perfusion system is a stable and robust method for assessing cardiac functional parameters, similar to the traditional open, constant-flow configuration. In addition, the recirculation option is advantageous for testing and screening molecules for cardiac and non-cardiac therapeutic purposes at an affordable cost, which should open up promising prospects for new drug identification and safety pharmacology studies. Therefore, their integration into multi- and interdisciplinary approaches combining computational methods with experimental and clinical approaches across academia, industry, and healthcare is expected to allow major advances in the prediction of cardiotoxicity in humans, both in health and disease [41].

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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