TRANSFUSION MEDICINE

Impaired O$_2$ unloading from stored blood results in diffusion-limited O$_2$ release at tissues: evidence from human kidneys

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KEY POINTS

- Slower O$_2$ unloading from stored blood reduces O$_2$ extraction in perfused kidneys, indicating diffusion-limited O$_2$ release at capillaries.

- Biochemical rejuvenation of kinetically compromised stored RBCs restores rapid O$_2$ release and raises renal cortex O$_2$ tension by 60%.

The volume of oxygen drawn from systemic capillaries down a partial pressure gradient is determined by the oxygen content of red blood cells (RBCs) and their oxygen-unloading kinetics, although the latter is assumed to be rapid and, therefore, not a meaningful factor. Under this paradigm, oxygen transfer to tissues is perfusion-limited. Consequently, clinical treatments to optimize oxygen delivery aim at improving blood flow and arterial oxygen content, rather than RBC oxygen handling. Although the oxygen-carrying capacity of blood is increased with transfusion, studies have shown that stored blood undergoes kinetic attrition of oxygen release, which may compromise overall oxygen delivery to tissues by causing transport to become diffusion-limited. We sought evidence for diffusion-limited oxygen release in viable human kidneys, normothermically perfused with stored blood. In a cohort of kidneys that went on to be transplanted, renal respiration correlated inversely with the time-constant of oxygen unloading from RBCs used for perfusion. Furthermore, the renal respiratory rate did not correlate with arterial O$_2$ delivery unless this factored the rate of oxygen-release from RBCs, as expected from diffusion-limited transport. To test for a rescue effect, perfusion of kidneys deemed unsuitable for transplantation was alternated between stored and rejuvenated RBCs of the same donation. This experiment controlled oxygen-unloading, without intervening ischemia, holding all non-RBC parameters constant. Rejuvenated oxygen-unloading kinetics improved the kidney's oxygen diffusion capacity and increased cortical oxygen partial pressure by 60%. Thus, oxygen delivery to tissues can become diffusion-limited during perfusion with stored blood, which has implications in scenarios, such as ex vivo organ perfusion, major hemorrhage, and pediatric transfusion. This trial was registered at www.clinicaltrials.gov as #ISRCTN13292277.

Introduction

Maintaining O$_2$ supply to tissues is a homeostatic priority and a primary concern in intensive care, transfusion, and transplant medicine. The canonical view[1] holds that gas exchange at capillaries is rapid, such that the O$_2$ partial pressures (P$_{O2}$) in the blood and surrounding tissues equilibrate by the time red blood cells (RBCs) reach the venous end of microvasculature. For example, blood P$_{O2}$ equalizes with alveolar P$_{O2}$ by a third of the length of pulmonary capillaries under resting cardiac output, which provides considerable reserve when blood flow

accelerates (e.g., exercise).[2,3] Rapid gas exchange is also assumed for systemic capillaries, although direct evidence is lacking. Consequently, O$_2$ delivery to tissues (D$_{O2}$) is considered a perfusion-limited process that can be modeled as the product of blood flow (Q), O$_2$-carrying capacity (K$_{O2}$) related to hemoglobin (Hb) concentration, and arterial HbO$_2$ saturation (S$_{A,O2}$), but independent of the kinetics of O$_2$-unloading from RBCs:

$$D_{O2} = Q \times K_{O2} \times S_{A,O2} \text{ (Equation 1)}$$

The assertion that O$_2$ exchange is not diffusion-limited has guided treatments for circulatory shock. Recommendations for

increasing D_{O_2} include positive inotropes (to increase Q), transfusion (to raise Hb content), and ventilation (to raise S_{A,O_2})[4] but not improvements to RBC gas-handling kinetics. Perfusion-limited delivery is embodied in early goal-directed therapy (EGDT),[5-8] the protocolized management of circulatory shock secondary to sepsis. In terms of physiological regulation, perfusion-limited O_2 delivery is favorable because the principal factor restricting O_2 supply to cells is arterial flow, which tissues control through vessel resistance.[9] For instance, if the respiratory demand for O_2 is not met, D_{O_2} can increase by raising Q. However, this linearity does not apply under a diffusion-limited scenario because increasing Q also shortens the capillary transit time, which reduces fractional O_2 unloading and offsets the effort to raise D_{O_2}. Thus, the kinetics of RBC O_2-handling are critical in determining the transition between perfusion- and diffusion-limited transport, but rarely measured. A commonly reported surrogate is Hb-O_2 affinity, but this steady-state variable cannot predict kinetics. For instance, 2,3-diphosphoglycerate (2,3-DPG) depletion stabilizes HbO_2, but whether this slows O_2 release to the diffusion-limited threshold is unclear. Indeed, a left-shifted O_2-saturation curve could still release the amount of O_2 instructed by respiration by attaining a lower P_{O_2} at the venous end, provided there is sufficient time for gas equilibration.

To measure O_2-unloading from RBCs, we developed single-cell oxygen saturation imaging. This revealed kinetics that are considerably slower than previous estimates[10] based largely on measurements from Hb solutions.[11-15] The additional delay is attributable to restricted gas diffusion across the macromolecule-packed cytoplasm. Consequently, changes in RBC shape can profoundly affect gas exchange. In storage, RBCs undergo metabolic and morphological changes[16,17] that result in a time- and donor-dependent attrition of O_2-unloading kinetics, reversible upon biochemical rejuvenation,[18] but whether this reaches the threshold for diffusion limitation is unclear. Multiple human trials and animal studies have suggested no negative impact of storage duration on transfusion outcomes,[19-22] although RBC O_2-handling kinetics were not recorded and, instead, randomization used storage duration, a metric that is not an accurate surrogate of RBC quality.[23] Moreover, those trials recruited participants with stable anemia, rather than patients requiring massive transfusion for whom the imperative is to restore O_2 delivery to tissues immediately and where diffusion-limited exchange would be problematic. Because transfused RBCs undergo rejuvenation upon recirculation in a physiological milieu, evidence for an effect of storage lesion should be based on recording responses shortly after transfusion.

To seek evidence for diffusion-limited O_2 delivery with stored blood, we studied ex vivo organs perfused entirely with stored RBCs and recorded tissue responses in real time, such as respiration and oxygenation. Red cell concentrates were obtained from the UK's National Health Service Blood and Transplant (NHSBT) in accordance with standard storage practice. The kidney is an appropriate organ for these experiments because arterial blood flow determines filtration rate, hence tubular transport and O_2 demand, and any discrepancy with arterial O_2 supply, as defined by Equation 1, would indicate diffusion-limited O_2 exchange (Figure 1A). These measurements were made during prolonged normothermic machine

perfusion of the kidney[24,25] conducted as part of a phase 1 trial testing the safety and feasibility of normothermic machine perfusion of the kidney prior to deceased-donor renal transplantation. In a second set of experiments, kidneys deemed unsuitable for transplantation were perfused alternately with RBCs of slow (stored) or fast (rejuvenated) O_2-handling properties to establish causality with tissue readouts. Because renal P_{O_2} gradients are relatively small owing to modest O_2 extraction (~10%-15%), any evidence for diffusion-limited delivery would likely apply to organs with greater fractional extraction, such as the heart. Compared with many other organs, kidneys show less variation in vascular function and are more readily available because transplants are common.

Our results indicate that renal respiratory rates were lower in kidneys perfused with RBCs of slow O_2-unloading kinetics. Consistent with a diffusion-limited process, a positive correlation between renal respiration and O_2 delivery is obtained once the rate of O_2 release from RBCs is considered. Switching to biochemically rejuvenated blood during perfusion promptly increased the kidney's O_2 diffusive capacity and cortical oxygenation. Our findings challenge the paradigm that O_2 exchange at capillaries is invariably rapid, highlight the need to consider RBC gas-handling properties, and justify efforts to optimize blood storage regimes or rejuvenation protocols.

Methods
Kidney perfusions
Normothermic Kidney Perfusion Phase 1 (NPK1) was a phase 1 regulated device trial (ISRCTN13292277; ethical approval was obtained from Greater Manchester South Research Ethics Committee REC 20/NW/0442) investigating the safety and feasibility of prolonged-duration normothermic machine perfusion of the kidney prior to transplant; the observational component of our study drew on samples and data from this trial. Additional ethical approval was obtained for experimental work with kidneys unsuitable for transplantation (REC 21/PR/1546). All participants in the clinical trial, blood donors, and donor families (for deceased-donor research organs) gave written informed consent. Organ retrieval procedures were conducted in accordance with normal clinical practice, and kidneys were transported to Oxford using conventional ice-box storage. Conventional back-table surgery was performed before attachment of the kidney to the investigational device and perfusion at 37°C with RBC-based perfusate before transplantation (Figure 1B). All units of blood used for perfusion were produced in accordance with NHSBT procedures, with cold-chain maintained until addition to the perfusion machine. During clinical kidney perfusions, blood gases were measured continuously (Terumo CDI-500) with additional arterial and venous point measurements made using an external blood gas analyzer (ABL-FLEX 90). The supply of O_2 and air was automated to maintain arterial P_{O_2}. Arterial pressure was automatically regulated (90 mm Hg for the first 24 perfusions and 75 mm Hg thereafter), as was temperature (37°C). For rejuvenation experiments, RBC units toward the end of their clinical shelf-life were split, and 1 part was treated with a solution of pyruvate, inosine, phosphate, and adenine (PIPA; marketed as Rejuvesol, Zimmer Biomet). Alternating perfusion between standard-stored blood and rejuvenated blood used 2 perfusion
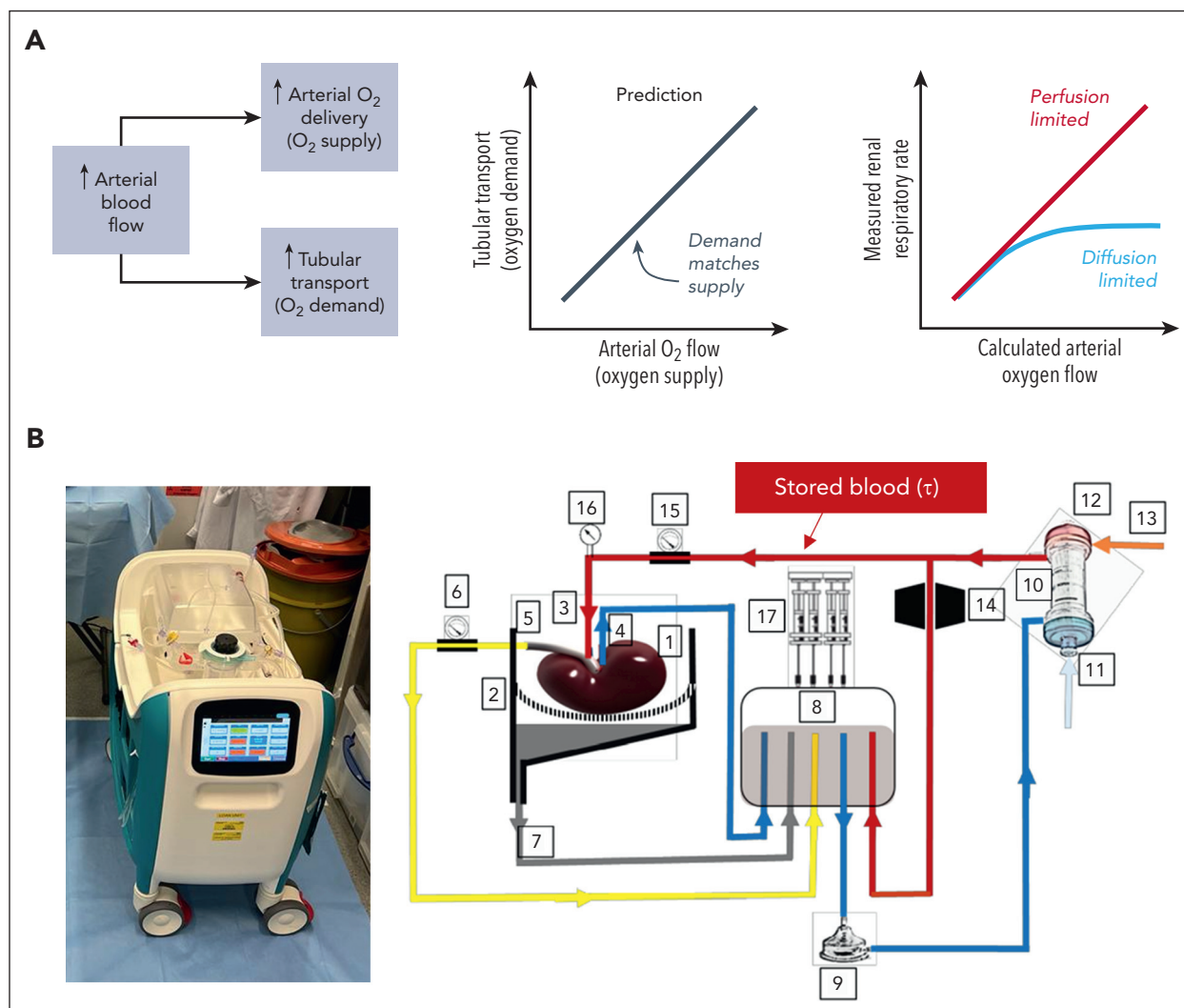
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Figure 1. Experimental design for testing oxygen delivery to perfused kidneys. (A) Higher arterial blood flow increases oxygen delivery to the kidney (O_2 supply). This also increases glomerular filtration and hence tubular reabsorption (O_2 demand). In a perfusion-limited scenario, O_2 supply and demand should be matched and a linear relationship is expected between arterial oxygen flow and renal respiration. Otherwise, if oxygen exchange from blood to the kidney was diffusion-limited, then the relationship between arterial oxygen flow and renal respiration is expected to tail off. Thus, it is possible to test for diffusion-limited oxygen exchange by relating measurements of renal respiration with arterial oxygen flow. (B) Image of machine for normothermic kidney perfusion and schematic diagram of the circuit in the kidney perfusion machine: 1, kidney; 2, organ containing with perforated kidney sling; 3, arterial cannula at kidney inlet; 4, venous cannula at kidney outlet; 5, ureter outlet duct; 6, urine flow meter; 7, duct for recirculation of fluids leaked by the kidney; 8, soft-shell reservoir; 9, centrifugal perfusion pump; 10, oxygenator and heat exchanger; 11, heat exchanger water inlet; 12, heat exchanger water outlet; 13, oxygenator has inlet; 14, in-line blood gas analysis sensor; 15, arterial flow meter; 16, arterial pressure sensor; and 17, infusion or syringe pump.

machines with separate blood loops feeding to a common dialysis system.

Single-cell oxygen saturation imaging

The kinetics of oxygen unloading from RBCs were studied using a modification of a previously published method.[10,26]

Results

Kidney perfusion generates data on renal function and respiratory rate

Thirty deceased-donor kidneys (mass, 140-410 g; median, 227 g) were obtained for normothermic machine perfusion with NHSBT stored blood of ABO-Rh type matched to the kidney donor. The duration of perfusion ranged from 2 to 23 hours, with a median of 5.8 hours. During perfusion, renal blood flow,

temperature, blood pressure, arterial pH, and urine flow were recorded continuously, and urine was collected for analysis. Regular point measurements of arterial and venous blood gases, biochemistry, Hb concentration, and O_2 saturation were taken by an external blood gas analyzer (supplemental Figure 1, available on the *Blood* website). Figure 2A and B summarize key data, including O_2 content of arterial and venous blood for calculating renal respiration. Blood flow stabilized within 1 to 2 hours of the start of perfusion (Figure 2C). Arterial pH started from 7.1 and gradually recovered over the first 3 hours of perfusion (Figure 2D). Renal respiratory rate, $v'_{R,O2}$, was calculated using the following equation (derivation provided in the supplemental Appendix):

$$v'_{R,O2} = Q_A \times (\alpha \times (P_{A,O2} - P_{V,O2}) + \beta \times [Hb] \times (S_A - S_V)) + Q_U \times \alpha \times (P_{V,O2} - P_{U,O2})$$
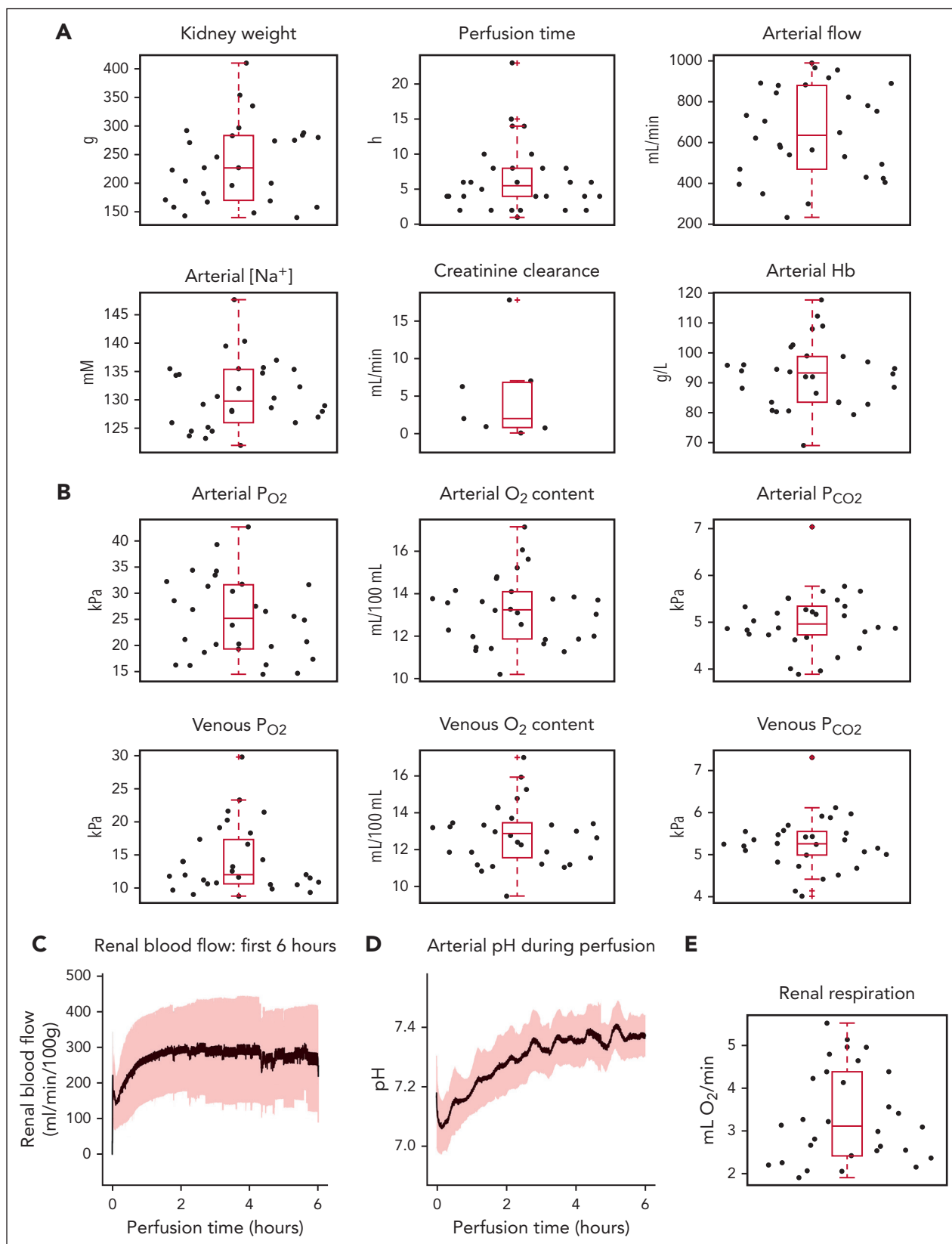
Figure 2. Normothemic kidney perfusion provides comprehensive data on renal function. (A) Data on kidney weight, perfusion time, time-averaged arterial blood flow, arterial Na^{+}, creatinine clearance, and arterial Hb. (B) Analysis of arterial and venous gases. (C) Time course of blood flow for first 6 hours of perfusion. (D) Time course of arterial pH for first 6 hours of perfusion. (E) Renal respiration calculated from blood gases.

$v'_{R,O2}$ varied from 1.9 to 5.5 mL/min O_2, with median of 3.11 mL/min O_2 (Figure 2E). The differences in $v'_{R,O2}$ were not due to variation in kidney mass (Pearson ρ = 0.10; P = .24).

Stored blood used for kidney perfusion manifested a range of kinetic dysfunction

The kinetics of O_2-unloading from RBCs were measured using single-cell oxygen saturation imaging under superfusion in a microfluidic chamber (Figure 3A) that allows rapid exchange between a normoxic and anoxic solution (Figure 3B). Owing to the O_2-dependent shift in Hb absorbance, the ratio of Cell-Tracker DeepRed and Calcein Green fluorescence describes the O_2 saturation of superfused RBCs. O_2 unloading was triggered by exposing RBCs to a 10-second period of anoxia (Figure 3C).

To obtain reference values for the O_2-unloading time constant, τ, freshly drawn venous blood was analyzed from 32 registered blood donors. Figure 3D shows their blood group and age. Mean corpuscular volume (MCV, median, 90 fL) and mean corpuscular Hb concentration (MCHC, median, 333 g/L) were normal. The median, was 0.975 seconds, consistent with previous recordings,[10,18] and the change in fluorescence ratio (O_2-carrying capacity, κ) was 0.499 (Figure 3E).

Next, measurements were performed on stored bloods allocated for kidney perfusions. Storage duration varied from 4 to 30 days, with a median of 12.5 days (Figure 3F). RBCs spanned a range of kinetic attrition, including RBCs with substantially slower O_2-unloading compared with reference blood (Figure 3G). Critically, the functional impairment incurred during storage did not correlate strongly with storage duration,[18] which indicates that storage duration is not a good surrogate for changes in RBC functional quality. This strengthens the case for correlating kidney outcomes with a direct measure of O_2 release from RBCs. Venous blood from the kidney recipient was also analyzed for RBC O_2-unloading and suggested a largely normal range with 2 outliers (Figure 3H).

RBCs circulating on the perfusion machine may become rejuvenated upon contact with the organ; thus, to relate renal respiration with RBC gas handling, small samples of blood were obtained during perfusions for measurements of τ and κ (Figure 4A-B). There was an overall trend for RBCs to reduce the rate of O_2 release and then undergo partial recovery. During this time, arterial pH also showed time-dependence, gradually recovering from mildly acidic to physiological levels. Thus, correlations between RBC O_2-handling properties and renal respiration used data once pH had stabilized, labeled here as "end point" (Figure 4C,D). Perfusion was determined to have a significant accelerating effect on O_2 release but, no significant effect on capacity (Figure 4E).

The ability of an RBC to release O_2 can be quantified in terms of its peak unloading rate, estimated from the time course of RBC oxygen saturation (S_{RBC}). The monoexponential curve that describes the time course of unloading is

$$S_{RBC}(t) = 1 + \kappa \times \left(e^{-\frac{t}{\tau}} - 1\right)$$

The time-derivative of this relationship is

$$\frac{dS_{RBC}(t)}{dt} = \frac{\kappa}{\tau} \times e^{-\frac{t}{\tau}}$$

Thus, the peak rate of oxygen release from the RBC ($v'_{RBC,O2}$) is

$$v'_{RBC,O2} = \frac{dS_{RBC}(t = 0)}{dt} = \frac{\kappa}{\tau}$$

Figure 4F plots calculated $v'_{RBC,O2}$ in stored blood before and during kidney perfusion, illustrating the considerable range in terms of ability to release O_2. If gas exchange were diffusion limited at capillaries, then release from RBCs at the lower range of $v'_{RBC,O2}$ is most likely to become rate-limiting for renal respiration ($v'_{R,O2}$).

Renal O_2 extraction from stored blood shows properties of a diffusion-limited process

In the kidney, higher arterial flow delivers more O_2 but also imposes a greater respiratory demand because of increased filtration and tubular transport. If O_2 transport were perfusion-limited, then the renal respiratory rate, $v'_{R,O2}$ is expected to be directly proportional to arterial O_2 delivery (D_{O2}) as calculated by Equation 1. If, however, O_2 delivery from stored RBCs becomes diffusion-limited, then linearity is not expected between D_{O2} and $v'_{R,O2}$. To that end, correlation was sought between $v'_{R,O2}$ and the canonical definition of D_{O2} that assumes a perfusion-limited process (D_{O2}^{PL}), defined as the product of arterial blood flow (Q_A) and arterial O_2 content ($C_{A,O2}$):

$$D_{O2}^{PL} = Q_A \times C_{A,O2}$$

D_{O2}^{PL} correlated strongly with arterial blood flow (Figure 5A), confirming that Q_A is the principal variable setting D_{O2}. However, there was no correlation between D_{O2}^{PL} and renal respiration $v'_{R,O2}$ (Pearson ρ = +0.359; P > .05; Figure 5B). This finding indicates that arterial O_2 flow is a poor predictor of the kidney's ability to extract O_2 to meet respiratory demands. A plausible explanation for this discrepancy is that O_2 exchange at capillaries becomes diffusion-limited for at least some perfusions, and is unable to provide the amount of O_2 needed to meet tubular transport demands. In support of this hypothesis, there was a significant inverse correlation between τ and $v'_{R,O2}$ (Figure 5C; Pearson ρ = −0.535; P = .00234). This finding indicates an effect of RBC gas-handling (cellular property) on renal respiration (tissue property). To test for a diffusion-limited process, correlations were sought between $v'_{R,O2}$ and a modified definition of arterial O_2 delivery (D_{O2}^{DL}) that includes a factor describing the initial rate of O_2 unloading from RBCs:

$$D_{O2}^{DL} = Q_A \times C_{A,O2} \times \frac{\kappa}{\tau}$$

Unlike the perfusion-limited definition, the diffusion-limited variant produced a strong correlation with renal respiration (Pearson ρ = +0.58; P < .0001; Figure 5D). The difference between these alternative models relates to the ability of RBC to release a flow of O_2 driven by a respiratory demand. Under perfusion-limited delivery, renal respiration extracts the required amount of O_2 owing to rapid O_2 exchange between
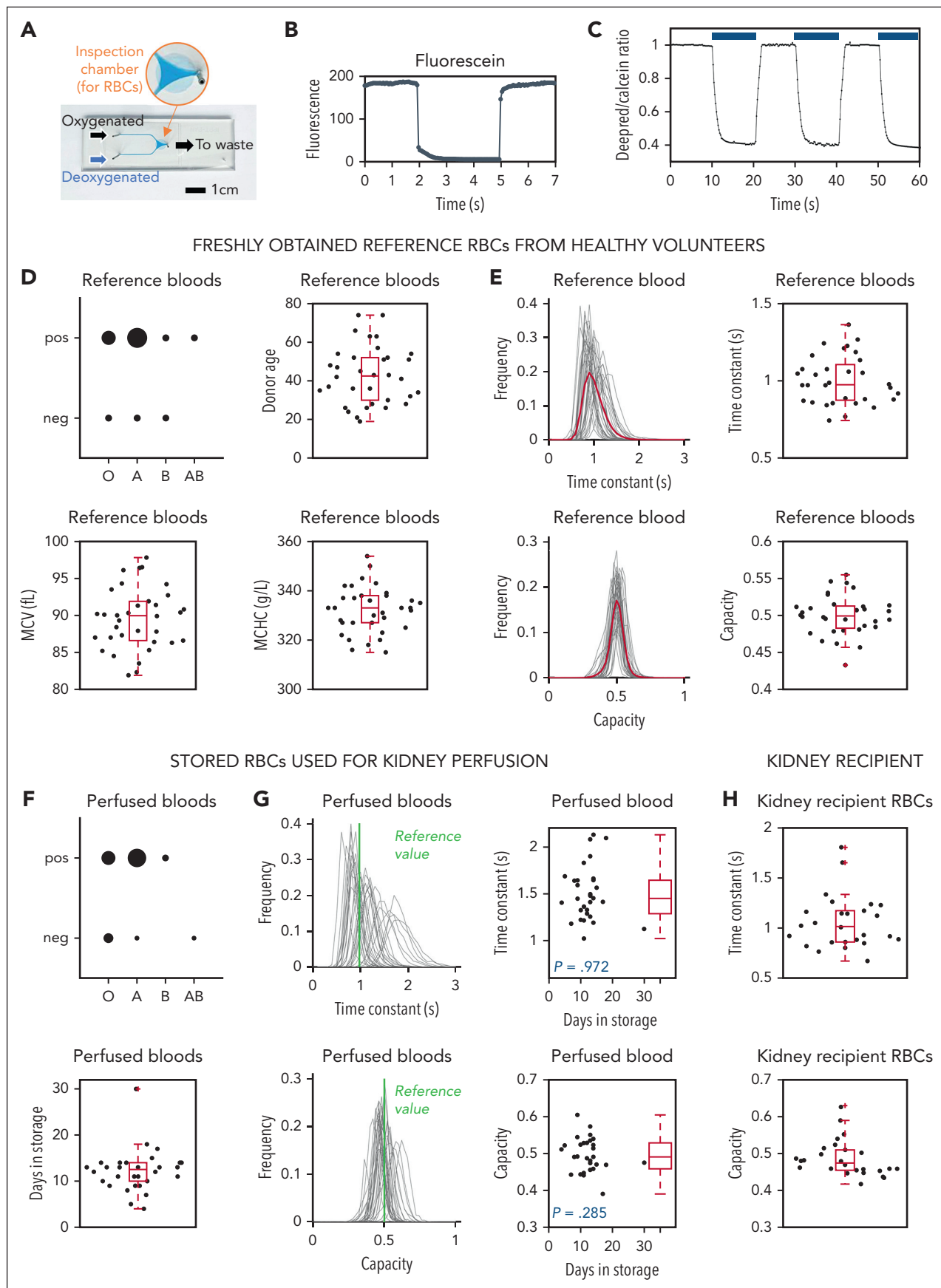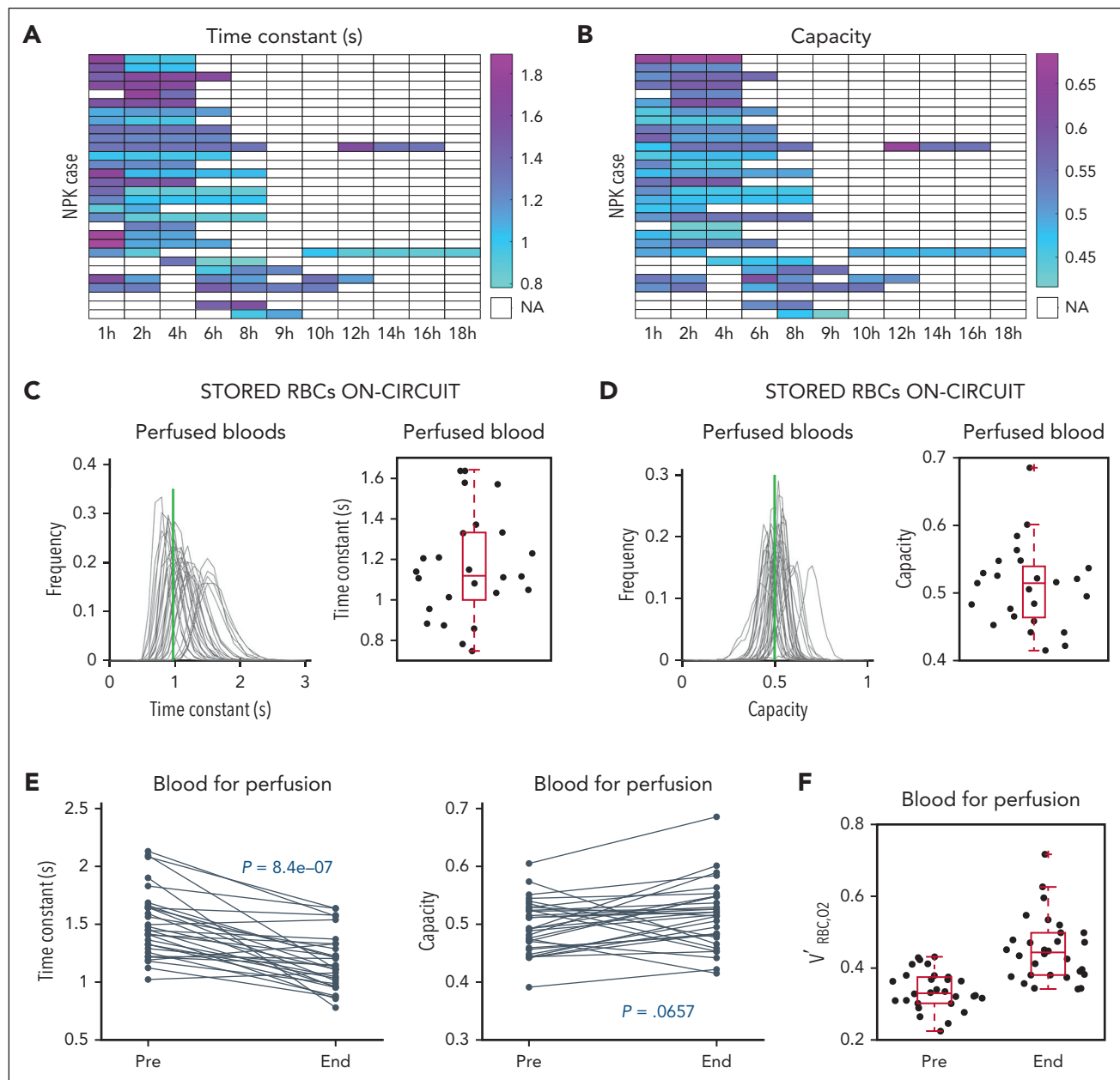
Figure 3.

Figure 4. Evolution of changes in RBC O₂-handling kinetics during kidney perfusion. (A) RBC O₂-unloading time constant measured at various points (x-axis) during perfusion for the 30 cases (along y-axis). (B) RBC O₂-unloading capacity measured at various points (x-axis) during perfusion for the 30 cases (along y-axis). (C) Analysis of stored blood at the end of perfusion. Time constant frequency distribution (green line shows reference blood mean). (D) Analysis of stored blood at the end of perfusion. Capacity frequency distribution (green line shows reference blood mean). (E) Effect of kidney perfusion on RBC oxygen-unloading time constant (significant decrease; paired t test) and capacity (no significant change; paired t test). (F) Initial oxygen-unloading rate calculated from the time constant and capacity. "End" denotes measurement towards end of perfusion; "pre" denotes packed RBCs prior to perfusion.

tissues and capillaries. Consequently, $\dot{v}'_{RBC,O2}$ becomes irrelevant, because full equilibration is imminent during capillary transit, and small variations in $\dot{v}'_{RBC,O2}$ would not affect tissue oxygenation. In a diffusion-limited scenario, the kidneys can only extract a fraction of the demand set by filtration and tubular transport because RBCs transiting capillaries will not

Figure 3. Single-cell oxygen saturation imaging characterizes RBC O₂-handling kinetics. (A) Microfluidic chamber for producing rapid solution exchange in imaged RBCs under superfusion. (B) Rapid exchange is achieved in the millisecond scale (frame acquisition: 51 ms). (C) Experiment on freshly drawn venous blood showing time course of oxygen unloading from imaged RBCs, quantified in terms of time constant (τ) and capacity (κ). (D) Reference blood freshly drawn from veins of healthy volunteers. Distribution by blood group, donor age, mean corpuscular volume (MCV), and mean corpuscular Hb concentration (MCHC). On each box, the central mark indicates the median; bottom and top edges indicate the 25th over 75th percentiles; the whiskers extend to the most extreme data points not considered outliers. (E) Analysis of reference blood. (Top) Time constant frequency distribution (red curve shows mean) and summary statistics; (bottom) capacity frequency distribution and summary statistics. (F) Stored blood used for perfusions. Distribution by blood group and distribution of storage time (days). (G) Analysis of stored blood. (Top) Time constant frequency distribution (green line shows reference blood mean) and summary statistics; (bottom) capacity frequency distribution and statistics. (H) Analysis of venous blood obtained from recipient of the kidney. (Top) Time constant statistics; (bottom) capacity statistics.
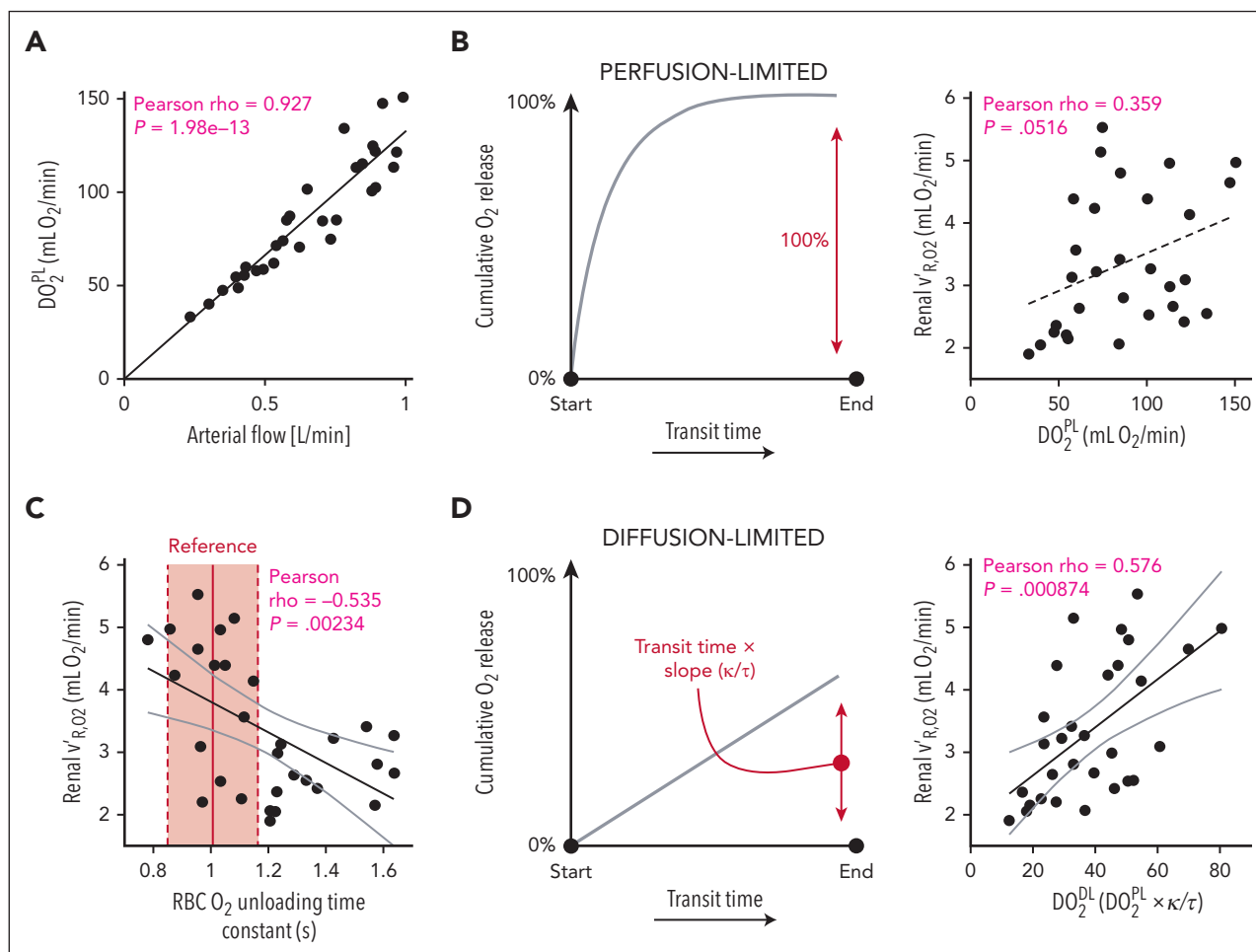
Figure 5. Relating blood-borne O₂ delivery with renal respiratory rate. (A) Strong linear relationship between arterial blood flow and arterial oxygen delivery calculated assuming perfusion-limited gas exchange. Statistical testing by the Pearson correlation coefficient. (B) Nonsignificant relationhip between arterial oxygen delivery ($D_{O_2}^{PL}$) and renal respiratory rate (v'_{R,O_2}), assuming perfusion-limited transport. (C) Strong, negative correlation between the RBC oxygen-unloading time constant and renal respiration (v'_{RO_2}). Reference range (red) determined from reference blood. (D) Strong, positive correlation between arterial D_{O_2} scaled by RBC initial oxygen-unloading rate (κ/τ) to model a diffusion-limited process, and renal respiration.

have enough time to equilibrate with tissue P_{O_2}. Consequently, renal respiration will be lower than expected for any given blood flow, explaining the inverse relationship, as presented in Figure 5B. The emergence of a strong positive correlation after introducing a factor describing RBC gas-handling (Figure 5D) indicates that gas exchange at capillaries is, indeed, diffusion-limited.

Rejuvenation of blood increases O₂ diffusion capacity and tissue oxygenation

The correlation between RBC O₂-handling properties and renal respiration is observational, but does not necessarily imply causality. Moreover, the kidneys represent a heterogenous pool of organs, which may affect correlations. Additional evidence for diffusion-limited O₂ release was sought by altering the O₂-handling properties of RBCs during perfusion of the same kidney. Two units of blood (ABO-matched and of equal storage duration within each pair, between 35 and 42 days) were pooled and split. One part was rejuvenated biochemically with a solution containing PIPA previously shown to improve oxygen-unloading kinetics,[18] whereas the other received sham treatment (i.e. with standard saline, adenine, glucose, and

mannitol containing additive solution). PIPA may not reverse all aspects of storage lesion, but its efficacy in restoring O₂ unloading is robust and directly relevant to our study. The component of PIPA with the greatest effect on accelerating O₂ handling is likely inosine, because omitting this substance abrogated rejuvenation efficacy (supplemental Figure 2A). Rejuvenated RBCs maintained faster O₂-unloading rates even when treatment was performed 2 weeks before measurements, giving a wide window of effectiveness (supplemental Figure 2B).

Kidney perfusion alternated between standard and PIPA-rejuvenated blood using a twinned dialyzed circuit that keeps all noncellular perfusate parameters and the respiring tissue constant, leaving RBC O₂-handling properties as the sole independent variable (supplemental Figure 3). Rejuvenated RBCs had faster O₂ unloading, without affecting carrying capacity (Figure 6A,C). The effect of perfusion with rejuvenated RBCs was quantified in terms of O₂ diffusion capacity, calculated as the ratio of respiratory rate v'_{R,O_2} to the P_{O_2} gradient between capillaries and tissue. Capillary P_{O_2} was approximated from arterial P_{O_2}, whereas organ-averaged tissue P_{O_2} was
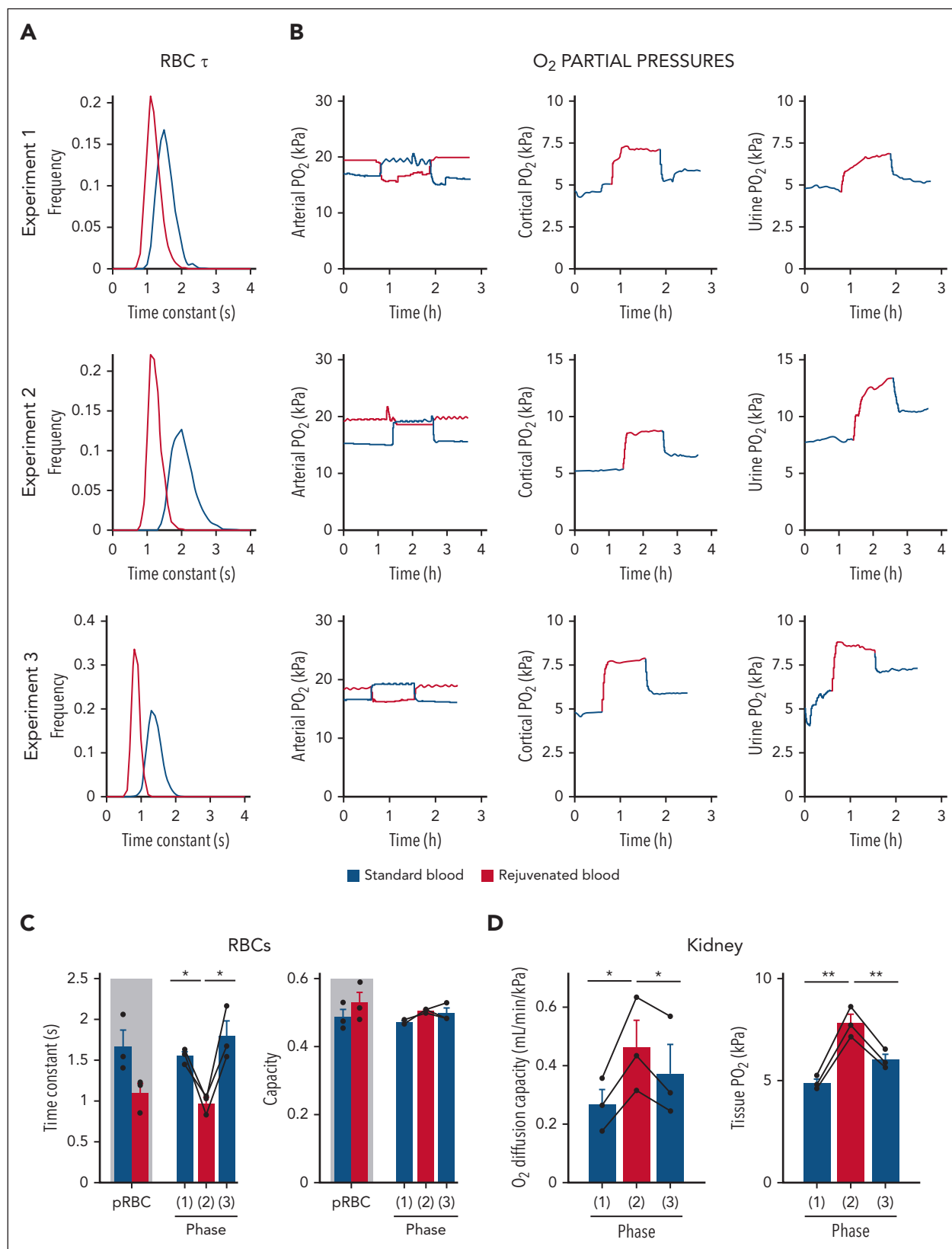
Figure 6. Evaluating the effect of biochemical rejuvenation on the ex vivo perfused kidney. Data shown for 3 independent experiments. (A) Single-cell oxygen saturation imaging of RBCs that had been biochemically rejuvenated or sham treated. Rejuvenation robustly restores the unloading time constant of long-stored RBCs toward reference values. (B) Matched time courses of partial pressures of O_2 (P_{O_2}) in arterial blood, kidney cortex, and urine during the 3 experimental phases: perfusion with standard (sham-treated) blood, rejuvenated blood, and back to standard blood. Significant improvements are observed in cortical and urine P_{O_2} during perfusion with rejuvenated red cells. (C) Oxygen-unloading kinetics (top) and its carrying capacity (bottom) measured in samples taken during the 3 phases of the experiment, in comparison to packed blood prior to perfusion. Significant effect of rejuvenation on kinetics during phase 2 (paired *t* test). (D) Oxygen diffusion capacity is significantly enhanced by rejuvenation, as is cortical oxygenation (paired *t* test).

approximated from urine P_{O_2} measured in the renal pelvis. Blood flow and urine production were constant during the experiment. If rejuvenation of O_2-unloading kinetics had a meaningful impact on gas exchange at capillaries (i.e., the system was diffusion-limited), then the O_2 diffusion capacity would increase during perfusions with rejuvenated blood, and reverse upon return to standard-stored blood perfusion. Faster O_2 diffusivity between RBCs and renal mitochondria would allow a smaller P_{O_2} gradient (i.e., higher tissue P_{O_2}) to drive an adequate O_2 flux that meets respiratory demand.

Figure 6B shows the time courses of P_{O_2} in the renal artery, cortex, and urine during this alternating perfusion protocol performed on 3 kidneys. Rejuvenation increased P_{O_2} in the cortex and urine, consistent with faster O_2 unloading having a favorable effect on overall diffusive capacity. This finding is in line with a diffusion-limited system. Notably, cortical P_{O_2} increased by 60% after the transition from standard to rejuvenated blood (Figure 6D). The diffusion capacity for O_2 increased significantly during perfusions with rejuvenated RBC, indicating that the rate of O_2 unloading from RBCs can meaningfully affect O_2 transport from capillaries to respiring cells. Because the microvasculature and energetic demand from tubular transport are constant during the experiment, the increase in diffusion capacity must relate to a change in RBCs, consistent with diffusion-limited transport.

Discussion

The results of our study highlight the circumstances that can lead to diffusion-limited O_2 exchange at systemic capillaries, and challenge the canonical view that this is invariably perfusion-limited. With an O_2-unloading time constant near 1 second in freshly drawn blood[10] and slowing further during storage,[18] it is possible that capillary transit times may not allow P_{O_2} equilibration.[27,28] Using human kidneys perfused with stored blood, we show that the attrition in RBC O_2-handling properties limits O_2 extraction driven by renal respiration. This relationship is unlikely to be affected by hypoxic signaling (eg, erythropoietin, EPO, production) because the duration of the perfusion periods in our interventional experiments precludes transcriptional responses. Arterial blood flow drives O_2 demand by setting tubular transport and also supplies O_2 for respiration; thus, a linear relationship between arterial O_2 delivery and respiration is expected under perfusion-limited transport. However, we observed no correlation between arterial O_2 delivery and renal respiration, unless the former included a factor describing O_2 release from RBCs, a defining feature of diffusion-limited exchange. Compelling evidence for diffusion-limited exchange was obtained by alternating the O_2-handling properties of RBCs during kidney perfusion. Here, the only factor being varied was the O_2-unloading time constant from RBCs, from slow, in standard-stored blood, to fast in its rejuvenated counterpart. Rejuvenation of stored blood hastened O_2 unloading and increased the O_2 diffusive capacity across the kidney, which allowed a smaller P_{O_2} gradient to drive the necessary flow of O_2 for respiration. Strikingly, a simple biochemical intervention performed on blood was able to increase cortical oxygenation by 60%. We conclude that O_2 release in the kidney becomes a diffusion-limited process at least in the case of RBCs manifesting a kinetic dysfunction, such as that incurred in storage. The emergence of diffusion

limitation in an organ, such as the kidney, characterized by a relatively small degree of O_2 extraction, makes it plausible that other organs, with higher extraction rates, also show such properties. Our results are consistent with an earlier report in rats receiving isovolumic transfusion of stored blood,[29] which found that blood samples stored for 5 to 6 weeks produced a greater decrease in renal microvasculature P_{O_2} than units stored for 3 days, but the effect of prolonged storage could be reduced by rejuvenation. All other factors being equal, a bigger drop in P_{O_2} is consistent with a greater diffusive barrier to the flow of O_2 between the blood and mitochondria. If RBCs are the independent variable, the most likely explanation is slower release in a diffusion-limited system.

Our findings have implications for our fundamental understanding of gas exchange. Contemporary models of blood transport should consider the possibility that at least under certain conditions, capillary P_{O_2} may not reach equilibrium with interstitial P_{O_2}, leading to a mismatch between demand and supply. If kinetically-compromised RBCs transiting capillaries have insufficient time to equilibrate P_{O_2}, then the cardiovascular responses that are normally effective in restoring oxygenation under a perfusion-limited scenario may not produce the desired outcome. Faster blood flow will further reduce transit time, and any increase in arterial delivery would be offset by incomplete fractional unloading of RBCs. Consequently, a diffusion-limited process is more likely to incur an oxygen debt, and the only way of improving delivery would be to address the rate-limiting process underpinning slow gas exchange in capillaries, that is, restoring RBC O_2-handling kinetics. In the case of pulmonary capillaries, diffusion-limited exchange may cause arterial hypoxemia, particularly under strenuous exercise. Indeed, there are reports of arterial hypoxemia in elite female athletes because of incomplete O_2 uptake by RBCs,[30] and any exacerbation of RBC kinetic properties would make this scenario more likely.

Diffusion-limited O_2 delivery with stored blood is predicted to reduce the maximal respiratory rate in exercise ($\dot{v}_{O_2}^{max}$). Attempts to test this have been made for elite cyclists receiving autologous transfusion with stored blood.[31] At least 1 study[32] showed that long-stored blood is less effective in improving exercise endurance compared with short-stored blood, which is consistent with our findings. Some studies have shown that $\dot{v}_{O_2}^{max}$ invariably increases with transfusion,[31] which could be considered as evidence against diffusion limitation. However, there are a number of factors to consider before reaching such a conclusion. Firstly, donor age and fitness may affect vulnerability to storage lesion, and it is plausible that elite cyclists undergo a less pronounced kinetic attrition in storage, compared with the general population. Because O_2-handling kinetics of RBCs were not measured in these studies, the significance of donor profile is unknown. Secondly, autologous transfusions will increase total Hb, which should increase O_2 delivery even if most of the transfused RBCs are kinetically inferior. This is because RBC O_2-handling shows considerable variation, and at least some cells will retain favorable properties, allowing $\dot{v}_{O_2}^{max}$ to rise, even if by a small but significant amount. Thus, both long- and short-stored blood are likely to increase $\dot{v}_{O_2}^{max}$, when total Hb is not controlled for. Finally, stored RBCs upon recirculation in a physiological milieu undergo a gradual restoration of key surrogates of storage lesion, including 2,3-DPG.[33] If $\dot{v}_{O_2}^{max}$ measurements are taken

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after in vivo rejuvenation is complete, the consequences of diffusion-limited exchange will not be apparent. Diffusion-limited O_2 delivery is most likely to emerge when the body attempts to increase tissue oxygenation by increasing blood flow under circumstances in which O_2-unloading from RBCs is impaired. Because cyclists are highly trained, their physiology may be well-adapted to handle an increase in blood flow within the limits of perfusion-limited delivery. A different situation will occur in patients who are critically ill, for whom attempts to increase blood flow could lead to diffusion-limited exchange after transfusion with stored RBCs. This may explain why there is no apparent benefit of liberal transfusion strategies over more restricted approaches.[34,35]

Although we have demonstrated diffusion-limited O_2 delivery during perfusion with RBCs compromised by the storage lesion, we have not shown its physiological consequence on the kidney, such as on signaling through hypoxia-inducible factor (HIF) and EPO production. In this study, it was critical to minimize bleeding to achieve technical success, which precluded taking repeated biopsies to examine tissue HIF induction. In our experiments, we sought to avoid possible on-circuit rejuvenation of RBC O_2-handling, which would be a confounding factor; therefore, our perfusions were shorter than the timeframe for HIF-evoked transcriptional responses. However, HIF stabilization and EPO production are aspects that should be investigated in future studies.

Evidence for diffusion-limited O_2 delivery has implications for clinical management of shock.[4] With diffusion-limited gas exchange, tissue oxygenation will not respond to inotropes because these would abbreviate transit time and reduce fractional release. Transfusions that aim to raise [Hb] should consider the O_2-unloading kinetics of the blood product and the speed with which a treatment effect is clinically required. In some circumstances (eg, top-up transfusions and no critical ischemia), kinetically-impaired transfused RBCs may gradually recover after transfusion and provide the intended benefit within hours. As a result, diffusion-limited O_2 transfer may not be apparent when considering clinically-relevant end points. However, when a treatment effect is required immediately due to critical ischemia or a high ratio of transfused product to native circulating volume (eg, ex vivo organ perfusion, major hemorrhage, large-volume pediatric transfusion for cardiopulmonary bypass, or repeated blood transfusions), slower O_2 release could offset the increase in O_2 content and not improve tissue oxygenation. EGDT uses various hemodynamic variables to guide the administration of fluids, packed red cells, inotropes, and vasopressors to increase blood flow, increase [Hb], and reduce the oxygen extraction ratio. Although early studies were promising,[5] a meta-analysis of 3 pivotal randomized, controlled trials indicated little benefit.[36,37] Systematic review and meta-analysis of studies specifically considering transfusion threshold have shown no benefit from transfusion.[38] Consequently, the International Guidelines for Management of Sepsis and Septic Shock recommend a restrictive transfusion policy.[39] It is plausible that a reason for the failure of EGDT to show more clinical benefit is that O_2 release from RBCs becomes diffusion-limited. Thus, the failure of transfusion to improve outcomes could relate to the kinetic attrition of stored RBCs, particularly when O_2 extraction is high. Beyond transfusion medicine, diseases that reduce oxygen release from RBCs, such as hereditary

spherocytosis, may result in severely diffusion-limited O_2 delivery to tissues.

Our findings highlight the importance of monitoring the kinetic quality of stored blood. We previously showed that the rate of kinetic attrition varies among donor units.[18] Storage duration itself is not an accurate proxy of kinetic quality and should be avoided when attempting to match units of stored blood to recipients, or when randomizing units for clinical trials studying transfusion efficacy. We propose that RBC quality should be determined using direct functional assays such as that presented here. When deemed necessary, blood should be rejuvenated biochemically to ensure that recipient outcomes are not compromised by problems associated with diffusion-limited gas exchange. Kinetic monitoring and biochemical rejuvenation may also extend the storage window for packed RBCs to help mitigate shortages of rarer blood types. Rejuvenation before blood product use is labor intensive, and we estimate a burden of 3 to 4 hours that may limit its use in settings, such as major hemorrhage. Although there is capacity to improve the formulation using τ as a performance readout, and the possibility of rejuvenating blood before issue, future studies should evaluate alternative storage regimes (eg, hypoxic storage[26]), the addition of phosphate and guanosine, or adjustments in pH. This may be particularly important in circumstances where the transfusion-load is a substantial fraction of the recipient's blood volume or when blood is given to alleviate critical ischemia.

It would be important to expand our study to other organs, such as the heart, brain, and skeletal muscle, where O_2 delivery must be as high as possible. Future trials should investigate how different storage protocols or rejuvenation techniques improve outcomes and test methods for measuring RBC kinetics so that donations can be matched to the most appropriate recipient.

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Authorship

Contribution: P.S. wrote the first draft of the manuscript, and all authors contributed to revisions and approved the final version; R.D. was the trial manager and lead researcher for NKP1; P.F. was the chief investigator; R.D., J.H., S.K., A.W., R.P., C.C., and P.F. were the trial management group, local investigators, designed the trial, and have access to the primary or identifiable trial data; R.D. conducted all clinical perfusions, assisted by J.F.; for single-cell saturation imaging and data analysis the diffusion-limited oxygen transfer hypothesis was developed by P.S., R.D., and J.R.; R.D. provided deidentified perfusate samples from NKP1;

RBC kinetic analysis was developed and conducted by P.S. and J.R., respectively; P.S., R.D., and J.R. performed data analysis; R.D., J.R., and P.S. developed the hypothesis-testing experiments for rejuvenation experiments; R.D. designed the twinned-circuit perfusion setup and performed the perfusions assisted by J.F. and J.R.; J.R. performed blood rejuvenation; R.D., J.R. and P.S. performed data analysis and interpretation of results; T.K., S.B., and P.K. provided research materials (microfluidics); and D.V., J.B., M.E., and C.C. engineered the perfusion machines and provided technical support.

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Footnotes

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